Flow injection analysis with bioluminescence detection

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FLOW INJECTION ANALYSIS
WITH BIOLUMINESCENCE DETECTION
by
MUSTAPPA BIN NAWA\-WI B.Sc., M.Sc.

A Doctoral Thesis
submitted in partial fulfilment of the
requirements for the award of
DOCTOR OF PHILOSOPHY
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Supervisor: Professor J.N. Miller
Department of Chemistry
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Untuk:
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dan....
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The detection of bacterial contamination of water, pharmaceutical products etc. is of great importance, and is most conveniently performed by the detection of bacterial ATP (Adenosine triphosphate) using the luciferin-luciferase bioluminescence system. This system uses unstable and expensive reagents, and emits transient light signals. In this study an FIA (Flow Injection Analysis) system was set up to monitor the light signal produced by the reaction. Using a luminometric detector (a liquid scintillation counter) with the FIA system, the reaction length, sample volume, flow rates, pH etc. were investigated.

Quick and simple methods of extracting bacterial ATP with the possibility of using an on-line system, were also investigated. These included surfactants such as cetrimide, Brij, Triton X-100 etc., ultrasonic treatment, lysozyme and a commercial extractant. These extraction methods were compared with conventional methods e.g. boiling tris-buffer.

Further, the effect of various drugs on these methods were examined. Most of the drugs tested were found to have no effect on pure ATP but apparently slightly reduced the ATP content of live cells. Tests have shown that this reducing effect is due to effect of the drugs on the live cells but not on the ATP. Thus interaction between the drugs and bacteria can be studied by the ATP firefly bioluminescence measurements.
INTRODUCTION

1.1 Chemi- and Bioluminescence

Chemiluminescence can be described as a process of light emission by chemical reaction. Not all chemically reacting systems which emit light are necessarily chemiluminescence; free illumination, electrically or mechanically induced emission produce incandescence. The energy liberated, produced by a highly exorogenic reaction, raises the temperature to the point of incandescence, resulting in emission. To differentiate between these two phenomena, chemiluminescence can be defined as a system which, when enclosed in a black body cavity, produces emission of a greater quantum intensity than would be emitted by the same black body at the same temperature at which the reaction is taking place.

Normally a black body reaction at temperatures below 500°C is not visible by the naked eye, therefore any visible radiation emitted by a chemical reaction below 500°C will be due to chemiluminescence.

Seliger and McElroy (1965) have defined two requirements for a chemiluminescence to occur:

1) Sufficient energy must be available in a single step
chemical reaction to enable the product or intermediate molecule to be in a first electronically excited state or vibrationally excited state.

2) In direct chemiluminescence, the product or intermediate molecule must be reasonably fluorescent so that activation by fluorescence is a probable event; or for sensitized chemiluminescence to occur, an efficient energy transfer must take place between intermediate and a second fluorescent species.

For an electronically excited state product to be formed, the geometry of the excited product is very important. If a configurational change occurs, the energy level of the excited molecule will be much higher than the reactant, chemiluminescence will not occur as shown in figure 1.1.

The scheme for the chemiluminescent reactions are as follows:

\[ A \rightarrow B^* \rightarrow B + \text{light (direct CL)} \]

\[ A \rightarrow B^* \]
\[ B^* + C \rightarrow B + C^* \]
\[ C^* \rightarrow C + \text{light (sensitized CL)} \]

( \( B^* \) and \( C^* \) are excited states)

The efficiency i.e quantum yield, of chemiluminescence reactions can be defined as the product of the chemical and
Non-chemiluminescent reaction with fluorescent products.

Chemiluminescent reaction

Rare chemiluminescent reaction.

A + B = Reactants.
C + D = products in ground state.
C' + D = products with C in excited state.

Figure 1.1 Electron transfer for a chemiluminescent reaction (Marcus 1965).
the physical efficiencies of the processes involved, and is given by the equation below:

\[ \Phi_{CL} = \Phi_R \times \Phi_{ES} \times \Phi_{FL} \]

where \( \Phi_{CL} \) = quantum yield (chemiluminescence)

\[ = \frac{hv}{\text{reacted moles of A/B}} \]

\( \Phi_{ES} \) = Efficiency for products of excited molecules

\( \Phi_{FL} \) = fluorescence efficiency

\( \Phi_R \) = fraction of molecules following the chemiluminescence reaction

The highest efficiencies are normally observed for bioluminescence, where for firefly reaction is close to unity. For inorganic chemiluminescence, the efficiencies rarely exceed 0.01 even for the brightest reaction.

Most chemiluminescent reactions are known to produce visible light emission with the exception of a few which emit ultraviolet light.
1.1.1 Chemiluminescence

Chemiluminescence other than bioluminescence can be classified into 3 categories:

1.1.1.1 Gas Phase Chemiluminescence

The first known gas phase chemiluminescence is the emission of light from the oxidation of white phosphorus in moist air. Ozone and atomic oxygen can react with oxides of nitrogen to emit light.

\[
\text{NO} + \text{O}_3 \rightarrow \text{O}_2 + [\text{NO}_2]^* \rightarrow \text{light}
\]

\[
\text{O} + \text{O} + \text{SO}_2 \rightarrow \text{O}_2 + [\text{SO}_2]^* \rightarrow \text{light}
\]

The combination reaction of sodium and chlorine in gas phase can also produce chemiluminescence (Rauhut 1969) as shown below:

\[
\text{Na} + \text{Cl}_2 \rightarrow \text{NaCl} + \text{Cl}^*
\]

\[
\text{Na}_2 + \text{Cl}^* \rightarrow \text{NaCl} + [\text{Na}]^* \rightarrow \text{light}
\]
1.1.1.2 Solid Phase Chemiluminescence

Cathode-ray tube and X-ray screen are examples of solid phase chemiluminescence. The number of solid substances which can undergo chemiluminescence is relatively small compared to other types of chemiluminescence. Other examples of solid chemiluminescence are siloxene (Isacsson and Wettermark 1974) and solid lithium organophosphide reaction with oxygen (Strecker et al 1973).

1.1.1.3 Liquid Phase Chemiluminescence

There are 4 most widely used luminescent molecules for analytical purposes; luminol (5-amino-2,3-dihydro-phthalazine-1,4- dione), lucigenin (bis-N-methylacridinium nitrate), lophine (2,4,5-triphenyl imidazole) and peroxalate.

Like all other chemiluminescent materials, these substances produce light in the presence of an oxidising agent and a catalyst. The reactions involved are shown in figure 1.2.

1.1.2 Analytical Applications of Chemiluminescence

Due to very low quantum yields, very few of these
Figure 1.2 Reactions of luminol, lucigenin, lophine and peroxysxalate
chemiluminescence reactions involving these materials can be used for analytical purposes. In gas phase chemiluminescence, nitrous oxide (Steven et al 1973), sulphur dioxide (Steven et al 1973 and Fontjin et al 1970) and ozone (Hodgeson et al 1970) have been used to monitor pollution. However, in liquid phase, a few compounds with slightly higher quantum yields have found many uses.

1.1.2.1 Direct and Coupled Reactions

The oxidation of luminol, lucigenin and lophine are strongly influenced by the presence of metal ions. Utilizing this property, methods have been developed for determination of cadmium(ii) and zinc(ii) (Burguera et al 1981), silver(I) (Steig and Nieman 1978) and cobalt(II) (Burguera and Townshend 1981).

Using catalytic property of iron porphyrin on the luminol reaction, Olieancz et al (1968) managed to quantify bacteria in water.

Many oxidase enzymes react with a substrate to produce hydrogen peroxide which can be detected by coupling these reactions to a subsequent chemiluminescence reaction. Bostick and Hercules (1975) have reported the determination of glucose in serum using luminol reaction with ferricyanide as a catalyst. The analyte react with glucose oxidase to generate hydrogen peroxide which then detected by luminol reaction.
This principle has also been applied by Williams and Seitz (1976) to determine lactate dehydrogenase activity by the following reaction:

\[
\text{NADH} + H^+ + \text{MB}_{ox} \rightarrow \text{NAD}^+ + \text{MB}_{red}
\]

\[
\text{MB}_{red} + O_2 \rightarrow \text{MB}_{ox} + H_2O_2
\]

\[
H_2O_2 + \text{CL reagents} \rightarrow \text{light}
\]

\(\text{MB} = \) methylene blue

\(\text{NADH} = \) reduced form of nicotine adenine dinucleotide

1.1.2.2 Chemiluminescence immunoassays.

One the most promising uses of chemiluminescence is in the immunoassay. It has been increasingly studied as it provides high sensitivity, rapid reaction and detection and is safe and non-toxic compared to radioimmunoassay (RIA) it has been increasingly getting more attention.

In this technique, chemiluminescent material is either used directly or indirectly as a label for quantitation of ligands which have been labelled with enzymes or chemiluminescent co-factors.

Several biologically important compounds have been successfully analysed by chemiluminescent immunoassay procedures using luminol and isoluminol as well as a peroxoaxalate compound.
Human IgG was assayed using luminol as the label (Hersch et al 1979). Progesterone was also labelled with luminol in an assay by Kohen et al (1979), while Schroeder et al (1981) labelled hepatitis B with an isoluminol derivative.

Mahant (1983) has described a novel method for analysis of human serum using bis-(2,4,6-trichlorophenyl) oxalate which was coupled to a flow injection system.

1.1.2 Bioluminescence

Bioluminescence is an enzyme catalysed chemiluminescence in which oxygen acts as an electron acceptor. Compared with conventional chemiluminescence, bioluminescence is a highly efficient luminescence, with quantum yield of around 100%. It is believed that the enzyme effectively channels the oxidative process to obtain a high quantum yield (Harvey 1952). This bioluminescence phenomenon is widespread in nature, common among animals as well as plants. The light emission can be intracellular or it may appear after compounds have been secreted or ejected outside the organism. Using this behaviour and mechanism observed in these animals and plants, several assay systems have been developed for biologically important compounds such as ATP, DPN, FMN, NADH and other co-factors.

There are several types of bioluminescence which can be classified according to their origins. The most important
is firefly bioluminescence which occur in certain insects.

1.1.2.1 Firefly Luminescence

Luminous insects occur in the families of Lampyridae, Elateriidae, Phenodidae, Drilidae and Rhagothalmidae (Harvey 1952), but the true fireflies belong to Lampyridae family. Though not all species in Lampyridae family emit light, most of the species have developed the capacity to emit light, in some emission it may only occur for a short time while others are luminous in the larval form only. There are also species where the female is luminous whereas the male is not. In those species of fireflies where both female and male are luminous, a fairly complicated signal system has been observed (Seliger et al 1964), it has also been accepted that these flash patterns are devices for attraction of the opposite sex.

The emission of light by these fireflies requires two substances; a substrate and an enzyme. The substrate luciferin and luciferase enzyme require ATP and magnesium to produce light. Dubois (Chase A. 1964) prepared two aqueous extracts, in hot and cold water, from fireflies' lantern, then upon mixing the two extracts together, luminescence occurred. From this experiment it was only after few years that firefly luciferase had been crystallised (Green and McElroy 1956). It was then followed
by crystallization of firefly luciferin by Bitler and McElroy (1957).

By reacting D(-)-cysteine with 2-cyano 6-hydroxybenzothiazole, White et al (1963) managed to synthesize d(-)-luciferin. If l(+) -cysteine is used instead of d(-)-cysteine, D(+) -luciferin will be produced. The structures of these substances are shown in figure 1.3.

Figure 1.3 The structures of oxyluciferin, D(-)-luciferin and dehydroxyluciferin.
1.1.2.2 The Firefly Reaction

The sequence of events during a firefly reaction can be shown in the following three reactions:

\[ E + LH_2 + ATP + Mg^{2+} \rightarrow E.LH_2 - AMP + PPI \] (1)

\[ E.LH_2 - AMP + O_2 \rightarrow E.P^* + CO_2 + AMP \] (2)

\[ E.P^* \rightarrow E.P + light \] (3)

The overall reaction can be written as:

\[ ATP + D-Luciferin + O_2 \rightarrow oxyluciferin + PPI + AMP + CO_2 + light \]

where PPI = inorganic pyrophosphate

E = enzyme (luciferase)

In the presence of luciferin, magnesium and ATP, luciferase catalyzes an initial reaction to form luciferyl adenylate bound to the luciferase enzyme and free phosphate. Following the formation of the enzyme-luciferyl adenylate complex, molecular oxygen then reacts with the complex resulting in the release of AMP, CO_2 and electronically excited oxyluciferin bound to the enzyme. McElroy et al (1969) had proposed this oxidation mechanism.
as shown in figure 1.4.

On returning to the ground state, the excited complex emits light leaving the product still bound to the enzyme. Finally, AMP and oxyluciferin are released from the enzyme and the enzyme is then free to enter another cycle of the reaction.

\[
\text{ATP} + \text{LH}_2 \rightarrow \text{PPi}
\]

\[
\text{AMP} + \text{P}
\]

\[
\text{LIGHT}
\]

Figure 1.4 Schematic mechanism of firefly luciferase reaction.

In an attempt to study the kinetics of the reaction, DeLuca and McElroy (1974), have found that when the two reactants were mixed together, the light intensity started to increase after a 25 millisecond delay, a maximum was then reached followed by a gradual decrease, see figure 1.5 and figure 1.6.
Figure 1.5 Time dependence of bioluminescence

The delaying period of 25 milliseconds which was observed at the beginning of the reaction had been suggested to be the rate limiting step of the reaction, which was probably due to a conformational change; the removal of a proton from luciferin before the oxidation.

A decrease in the availability of free enzyme has resulted in the decrease in the intensity after the maximum is achieved. The unavailability of the enzyme is brought about by the strong binding of oxyluciferin and adenosine monophosphate (AMP) to the enzyme. The strong binding between the enzyme and the products has resulted in a
formation of a very strong complex. Gates and DeLuca (1975) studied this complex and managed to isolate it in the presence of pyrophosphatase by gel filtration on Sephadex G-25.

In addition to the inactivation of luciferase by the formation of the complex, AMP and oxyluciferin are also competitive inhibitors of luciferase.

Figure 1.6 Light output by different enzymes

The kinetic of light emission using crude luciferase prepared from firefly lantern extract is rather complicated due to the presence of ATP converting enzymes such as
adenylate kinase, nucleoside diphosphate kinase and creatine kinase (Rasmussen and Nielsen 1968). These enzymes contaminating continuously change the ATP level in the system causes an unpredictable emission of light. With purified luciferase (Lundin et al 1976), containing no ATP converting contaminants, the rate of decay in light emission can be drastically reduced and maintained constant with time (see figure 1.6).

1.1.2.3 Other Bioluminescence

Bacterial bioluminescence:— Most types of luminous bacteria are found in marine environments. There are four major types occurring in the ocean, with three classified as symbionts of fish. As in the firefly, the glow from these bacteria are also considered to be produced from a specialised light organ contained in the host cell, supporting their behavioural and survival purposes.

These bacteria fall into two categories:—

i) vibrio
   eg. vibrio harveyi, vibrio fischeri

ii) photobacterium
   eg. photobacterium phosphoreum, photobacterium leiognathi

The mechanism of these luminescence are said to be the same for all bacteria. Most of the time these bacteria devote
their metabolic activity towards the generation of photon. Strechler (1953) was first to study the mechanism of light emission and found that NAD was needed. A later study by Strechler et al (1954) found that FMNH₂ was the active co-enzyme generated from the enzyme catalyzed reduction of FMN by NAD(P)H. Another factor needed is a long chain fatty aldehyde (McElroy and Green 1955). An extract from vibrio fischeri was later found to increase the light by 100%, this was then called "bacterial luciferase" (Hasting et al 1965).

The bacterial luciferase system has been used extensively for assaying reactions involving pyridine nucleotides in many biological fluids. The reaction used for analytical purposes is as follows:

\[
\text{NAD(P)H} + \text{H}_2 + \text{FMN} \rightarrow \text{NAD(P)} + \text{FMNH}_2
\]

\[
\text{FMNH}_2 + \text{O}_2 + \text{RCHO} \rightarrow \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{light}
\]

The reaction between \text{NAD(P)H} and \text{FMN} is catalysed by \text{NAD(P)H}, \text{FMN oxidoreductase} to produce \text{FMNH}_2, this will then react with long chain aldehyde in the presence of oxygen catalysed by luciferase to produce light.

Theoretically any reaction which involves the appearance or disappearance of reduced pyridine nucleotides can be utilised for analysis. By making \text{NAD(P)H} the limiting component, the light intensity produced by this reaction is
directly proportional to the concentration of NAD(P)H.

The enzyme involved in this reaction can be assayed individually or in a coupled reaction. Oxidoreductases can be monitored spectrophotometrically at 340nm while luciferase can be assayed by reacting excess FMNH₂, aldehyde and oxygen. Hasting et al (1978) showed that when all of the substrates are present in a saturated amount, the light intensity is proportional to the product of the concentration of the two enzymes, a range of 10⁻⁹ to 10⁻¹⁶ mol (Stanley 1971) and a detection limit of 10⁻¹⁶ mol NADH can be achieved (Lee et al, 1974).

Besides its use for quantitation of NADH and NAD(P)H, the bacterial luciferase has also been used to assay flavin nucleotides (FMN). Wettermark and Brolin (1969) detected FMN using luciferase from photobacterium fischeri this method has also been used to determined glucose, malate and NAD using appropriate enzymes for conversion to NADH. Trinitro toluene (TNT) has also been assayed using TNT reductase (Gawronski and Egghart 1979).
1.1.2.4 Factors affecting luminescence

i) pH

A study by Lundin et al (1976) has shown that the optimum pH for firefly reaction is 7.75. A yellow-green light is emitted with a peak maximum at 560 nm. At lower pH eg. pH 6.0, the activity of the luciferase is only about 8% of that obtainable at the optimum pH. This behaviour shows that the enzyme luciferase is extremely sensitive to any change in pH. For normal analytical purposes, the pH range 6.5 to 8.5 has been recommended, provided that the buffering capacity is not exceeded.

ii) Temperature

Like all other enzymic reactions, the rate of the luciferase reaction is temperature dependent as shown by Myhrman et al (1978). The optimum temperature for this reaction is 25°C. At 20°C and 30°C the efficiency of the luminescence is only about 75% of that obtained at the optimum temperature. At higher temperatures the enzyme can be denatured and the activity reduced accordingly. A reversible red shift in the emission spectrum was observed when the temperature was increased above 25°C (Seliger and McElroy 1964).
iii) Solvent

In solvents other than water and in high ionic strength solution, the reaction behaves rather differently. This is due to the structural differences in the product emitter i.e. oxyluciferin. White et al (1969) found that in organic solvents, electronically excited oxyluciferin is in the form of a monoanion compared to a dianionic form in water. The structures are shown in figure 1.7.

\[
\begin{align*}
\text{Red light} & \quad \text{yellow-green light} \\
\end{align*}
\]

Figure 1.7 Structure of monoanion and dianion forms of the oxyluciferin molecule.

iv) Inteferences

ATP-converting enzymes in unpurified luciferase are a serious source of interference in the reaction as mentioned in the previous section.

The luciferase reaction has also been found to be inhibited by salts. It has been suggested that some anions inhibit the reaction by causing a small conformational change in
the active site of the luciferase (Denburg and McElroy 1970). It was also found that SCN⁻, I⁻, NO₃⁻,Br⁻ and Cl⁻ inhibit the reaction SCN⁻ having the greatest effect on the reaction. Gilles et al (1976) found ClO₄⁻, I⁻, Cl⁻ and acetate suppress the reaction with acetate being the least effective. From these findings it has been recommended that all of the salts used in the buffers should contain either chloride or acetate as the anion causes the least inhibition to the firefly reaction.

1.1.2.5 Analytical applications of firefly bioluminescence

The analytical applications of firefly luminescence are mostly concentrated around the use of ATP as an analyte. As ATP is contained in living cells, it may be utilised in measuring bacterial growth and counting bacterial numbers.

Since ATP plays an important role in the metabolism of a living cell, is present in high concentration compared with other metabolites, and is also uniformly distributed in the protoplast, it can reflect the number of cells present.

1.1.2.5.1 Biomass Determination and Bacterial Enumeration

The cellular ATP may readily be extracted from the bacteria. Bacteria have a high turnover of ATP and ATP is rapidly lost from dead cells. These factors provide a good index for cell viability. The number of cells that can be
detected by the firefly assay depends on the size and ATP content of the cells. Hamilton and Holm-Hansen (1967) and Chappelle and Levin (1968) have indicated that the ATP level in bacteria can be directly correlated to the number of cells. The content differs from one species to the other as shown in the table 1.1.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>ATP content(µg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobacter aerogenus</td>
<td>0.28 X 10^{-10}</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>1.10 X 10^{-10}</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1.00 X 10^{-10}</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>5.00 X 10^{-10}</td>
</tr>
<tr>
<td>Micrococcus lysodeikticus</td>
<td>1.30 X 10^{-10}</td>
</tr>
<tr>
<td>Mycobacterium phlei</td>
<td>1.90 X 10^{-10}</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1.00 X 10^{-10}</td>
</tr>
<tr>
<td>Psedomonas fluorescens</td>
<td>3.10 X 10^{-10}</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.64 X 10^{-10}</td>
</tr>
</tbody>
</table>

Table 1.1 ATP content of various bacteria.

With basic commercially available instrumentation and reagents, the detection limit for ATP is 10^{-10}µ which would correspond to about 10^6 to 10^8 cells/ml, however the possibility of measuring a single cell in solution has been demonstrated by Wettermark et al (1975).
1.1.2.5.2 Bacterial Adherent measurement

The adherence of bacteria to mucosal surfaces has been implicated as a factor in infection in a number of diseases such as urinary tract infections, respiratory infections, gastrointestinal infections, and gonococcal venereal disease. Harber et.al. (1982) have developed an in vitro method for quantifying bacterial adherence to urinary tract by firefly bioluminescence. The ATP was measured after removing non-adherent bacteria by washing, then treating the adherent bacteria with a commercial nucleotide releasing agent.

The adherence and accumulation of oral streptococci to tooth enamel produces dental plaque. Robrish et.al (1978) and Distler (1980) managed to measure ATP in monkey and human teeth by this method, using the boiling tris extraction method. Kemp (1979) converted ADP and AMP to ATP before measuring the total ATP, giving a better estimate for viable cells in dental plaque.

1.1.2.5.3 Bacteriuria screening test

The presence of Escherichia Coli in urinary tract indicates an infection. Screening methods for these bacteria have been developed by Piccolio et.al (1975), Thore et.al (1975) and Johnston (1976). Urine is a very complex matrix containing bacterial as well as non-bacterial cells. In
this method non-bacterial cells have to be lysed first, usually using Triton X-100 before extracting bacterial ATP. Conn et.al (1975) and Johnston and Curtis (1979) indicated that false negative results would be obtained due to incomplete destruction of the non-bacterial cells and suggested the treatment using Triton and apyrase before the extraction of ATP.

1.1.2.5.4 Vitamin and Antibiotic Assay

The cellular ATP levels of bacteria can be used as an index of growth of the cells. The presence of growth-promoting or growth-inhibitory substances such as vitamins and antibiotics will be reflected in the ATP content in the cells. Based on this principle, Haber and Assher (1977, 1979) have developed an assay method for gentamicin and folic acid.

Nilsson (1978) demonstrated that, for determination of gentamicin, the extraction procedure can be eliminated. After 75 minutes incubation of E.Coli in a nutrient containing serum and gentamicin, ATP leaks from the cell into the surrounding solution. The extracellular ATP can then be determined.

1.1.2.5.6 Determination of Enzymes and Metabolites.

As suggested by Strechler and Totter (1952), all enzymes and metabolites participating in the ATP reaction can be
determined by the firefly bioluminescence assay. This can be achieved by measuring aliquots of an ATP-converting reaction. The technique is based on either the formation or degradation of ATP.

Based on the conversion to ATP, Lundin et al (1976) determined four metabolites, i.e. ATP, ADP, AMP and cAMP in a single analysis by stepwise addition of appropriate enzymes.

McCoy and Doeg (1975) also using the formation of ATP as a basis for the determination of phosphophenolpyruvate (PEP). The PEP solution or an extract containing the PEP was incubated with pyruvate kinase and ADP and the resulting ATP formed by the reaction was then detected by firefly bioluminescence.

Cavari and Phelps (1977) used the degradation reaction of ATP by hexokinase to determine glucose.

Many enzymes can be determined using the firefly reaction such as ATPase (Strehler 1968), adenylate kinase (Brolin et al 1979) and phosphodiesterase (Fertel and Weiss 1978).

1.1.3 Advantages and Disadvantages of Luminescent Assays.

1.1.3.1 Intrinsic features

Bioluminescence reactions produce their own light requiring
no external light source thus giving a much lower background than in other methods such as fluorescence and absorbance. As described in chapter 2 this feature is an advantage in a flow system as there is no disturbance in signal with the introduction of air or air bubbles in the flowing stream.

1.1.3.2 Biological Occurrence

Most bioluminescence reactions require widespread and important reactants such as ATP, NAD(P)H and FMNH₂. As these substances exist in the living organisms and can be taken as an indication of its living activity, bioluminescence provides an essential tool for the study of biochemical and physiological characteristics of living creatures. However, the availability of these compounds in pure form is the biggest limitation. Generally, the luminescent organism has to be collected in the field and processed quickly, normally on site. The yields from these extractions are very low which makes the commercially available material slightly more expensive.

1.1.3.3 Sensitivity

The high quantum yield of bioluminescence reactions contributes to the high sensitivity of this method. With the availability of sensitive measuring instrumentation, i.e., a luminometer and the relatively fast enzymic reaction, a detection limit of $10^{-15}$ for ATP (Wannlund et al. 1982)
and $10^{-14}$ M for FMNH$_2$ (Lee et al. 1974) have been reported. The sensitivity of this method is about $10^{10}$ times that of the spectrophotometric methods and $10^6$ times of the fluorimetric methods. The limit of this sensitivity is only imposed by the background emission or the blank.

1.1.3.4 Linear response

The linear response of luminescence method is about 4 to 7 orders of magnitude. In the case of an ATP assay by firefly bioluminescence, the linear response is over 6 orders of magnitude. Normally linearity starts from the minimal detectable concentration of the compound up to the point where it is no longer possible to maintain an excess of the reactant relative to the analyte.

1.1.3.5 Specificity

Most bioluminescence reactions are specific because they are enzymic processes involving cofactors eg. ATP. These cofactors are also not susceptible to contamination or interference. However chemiluminescence reactions are non-specific, also the reactions are subjected to interference by reducing agents.

1.1.3.6 Speed

The speed in which a luminescence can be prepared depends upon the type of reaction. Some reactions produce a rapid
flash with a peak height proportional to analyte concentration while others will give a protracted glow lasting several minutes. In this type of reaction reproducibility suffers and the results are subjected to repetition and statistical analysis. In the ATP bioluminescence reaction, with purified enzyme, a quick and sustained flash is produced giving a quick and reproducible analysis.

1.1.3.7 Cost

The extremely high sensitivity of bioluminescence and the speed of its reaction allows a reduction in the amount of the reagent and the time consumed; thus reducing the cost per test.

1.1.3.8 Stability

Chemiluminescence reagents, such as luminol and lucigenin are quite stable at room temperature, whereas bioluminescence reagent suffer from instability as is normally encountered with many other enzymes. Firefly luciferase has to be kept at -4°C during use and -18°C for longer storage.

1.1.3.9 Toxicity and Hazard

To date, bioluminescence compound present no hazard to mankind and the environment. They can provide an
alternative assay to radioisotope technique in some analyses.

1.1.4 Instrumentation

One of the principle advantages of chemiluminescence and bioluminescence methods is the modest instrument required. A basic instrument requires only a light detector, a cell and an electronic readout. This simple chemiluminescence detector could easily be constructed with a minimum cost and other instruments can be adapted without highly sophisticated electronic modification. Compared to spectrophotometer and spectrofluorometer, a luminometer is the simplest instrument as shown in figure 1.8.

1.1.4.1 The Detector

The photoelectric detector is the most widely used detector for measuring the light produced by chemiluminescence reactions. In a more sensitive instrument, a photomultiplier tube consisting of several internal photocathodes, amplifying the signal from a single electron are employed. The drawback of photomultiplier tube is that its spectral range is only about 350nm to 450nm, while most of chemiluminescence and bioluminescence emissions occur at wavelengths higher than 450nm. A red sensitive photomultiplier tube is suitable for use at wavelengths
Figure 1.8 Comparative analytical techniques of colorimetry, fluorimetry and luminometry.
around 500nm, but it needs cooling to avoid a high background reading.

For a less sensitive instrument, solid-state detectors (silicon photodiodes) are used, providing a spectral range between 420nm to 600nm with ruggedness, portability and rapid warm-up.

1.1.4.2 The Cell
The requirements for the cell are that they are optically reproducible and that they can be reproducibly positioned in the cell holder. Scattering and reflection of light pose no problems in the measurement of light from a chemiluminescence reaction. In a batch analysis, disposable cells are normally used, the variation in the optical properties of each cell may contribute to any imprecision observed. For a flow analysis, glass, teflon or polyethylene tube can be used. This reduces the problem associated with the cell differences found in the batch analysis.

1.1.4.3 Detector chamber

The detector and the cell are housed in a detector chamber. It is normally designed to prevent extraneous light from reaching the detector and at the same time optimizes the collection of light by the detector. For a greater efficiency of light collection, a reflective surface (figure 1.9) is used on the other side of the detector to deflect
Figure 1.9 Diagrams of reflection for efficient light collection in a detection chamber of i) a luminometer ii) liquid scintillation counter.
light emitted in its direction.

1.1.4.4 Electronic readout

An electronic setup which would allow transformation of signal detected by a photodetector into a readable digitized or analogue form is quite adequate in a simple instrument. For a more sophisticated instrument, a microprocessor which would allow a vast range of activities, such as automatic injection, multiple readings and variable delay. It could also be used to perform standard curve fitting and all relevant calculations on the results.

1.1.4.5 Modified instruments

1.1.4.5.1 Liquid scintillation counter

A liquid scintillation counter is a two channel photon counter with a variable detector. For measuring a chemiluminescence signal, the coincidence feature is normally disabled, so that it can be used either as a single photon counter or using both counters in a non-coincidence mode, thus doubling the counting rate.

The problem associated with liquid scintillation counter is that most of the instruments available are designed for batch analysis, i.e the reaction has to be carried out outside the instrument, then placed in the detector often
via a conveyor belt. Another limitation is that it can only be used for low level detection. At this level the signal in coincidence mode is proportional to intensity squared.

1.1.4.5.2 Spectrophotometers and spectrofluorometers

Spectrophotometers and spectrofluorometers can be used for chemiluminescence measurement if the source can be turned off or the entrance slit closed while the detector remains active. Compared to a liquid scintillation counter, the efficiency and sensitivity are normally much lower.
1.2.0 Flow Injection Analysis

Flow injection analysis (f.i.a.) was introduced by Ruzicka and Hansen (1975) as a new analytical technique for the analysis of samples in a non-segmented flowing stream. This technique is apparently similar to the technique of non-segmented continuous flow and high performance liquid chromatography (h.p.l.c.). The difference is the precise requirement of the unsegmented stream, reproducible sample injection and controlled dispersion. The steady state signal is not required in an f.i.a. compared to other method. The different requirements are shown in figure 1.10.

In an air segmented continuous flow system as described by Skeggs (1957), the sample is added in alternation with wash solution to a reagent line, usually as a broad slug; the combined stream of reagent and the sample is then segmented by air bubbles and then flow through the incubation coil, then into the detector. The signal recorded by the detector is a curve that reaches a plateau, the height of which is proportional to the analyte concentration. The complexity of the introduction of air bubbles and the rate of sample throughput are just two of the disadvantages of an air segmented system over an f.i.a. system.
a) High Performance Liquid Chromatography

b) Air segmented continuous flow.

c) Flow Injection Analysis.

Figure 2.1 Schematic comparative of various principles.
In an f.i.a. system, the sample is injected as a plug into reagent stream and flows through a mixing coil and then into the detector. A series of peaks are obtained with the height of each peak being proportional to the corresponding analyte concentration.

The time taken for each discrete sample is minimised as is the reagent and sample consumption. The dispersion of the sample or reagent can be controlled to suit the detection method and the chemistry associated with it. The comparison and advantages of f.i.a. over the air segmented method have been discussed by Synder (1976) and Patton and Crouch (1986).

Since its introduction, f.i.a. has been developed extensively and expanded very rapidly. There have been three international conferences solely devoted to f.i.a..

1.2.1 Principle of f.i.a.

Figure 1.11 A typical single channel f.i.a. manifold
Basically an f.i.a. system consists of a pump, injection valve, mixing coil and detector as shown in figure 1.11. The sample is introduced through the injection valve as a plug then propelled by a carrier or reagent stream. The then reacts with the reagent to form a compound which can be detected as it passes through the detector.

1.2.1.1 Dispersion

![Diagram of dispersion in f.i.a.](image)

Figure 1.12 Definition of dispersion in f.i.a.
Dispersion $D$ has been defined as:

$$D = \frac{C^o}{C_{\text{max}}}$$

where $C^o$ is the original concentration of the sample

$C_{\text{max}}$ is the concentration of sample at the peak maximum at time of detection

Since in most of the f.i.a. determinations, the analytical method readout is peak height, which is proportional to the concentration of the sample, the dispersion $D$ can be expressed in peak height term as:

$$D = \frac{H^o}{H}$$

where $H^o$ is the peak height obtained from the original concentration of the sample

$H$ is peak height obtained when a volume of the sample is injected into the carrier stream

The value of $D$ reflects the amount of dilution that has taken place before the sample reaches the detector. A high value of $D$ means a high dispersion, thus high dilution of the sample in the carrier solution and vice versa.

A variation in the degree of dispersion of a sample in an f.i.a. system can be obtained by changing the inner
diameter of the tube, the length of the coil, the pumping rate of the streams and the volume of sample injected. Using small-bore tubing will decrease the dispersion, but there are limitations such as the blockage of the tube by particles in the flowing streams, which increase the pressure resulting in a very high back-pressure.

The dispersion increases with the square root of the tube length, thus, lengthening the reaction coil is an efficient way of promoting mixing in an f.i.a. system.

The flow-rate has very little influence on the dispersion. Increasing the flow rate will only slightly increase the dispersion.

A better method of changing the dispersion is to alter the sample volume. Dispersion decreases with the increase in the sample volume giving rise to peak height thus the sensitivity of the measurements.

Another efficient method of increasing the dispersion is to merge two streams as in the merging zones method.

1.2.1.2 Residence Time

Assuming that the flow of the stream is constant and free from air bubbles, the residence time assumed to be as always constant. During this period, the reaction between
the analyte and the sample takes place and a detectable product is formed. Since this residence time is constant, it is not necessary that the reaction reaches completion, which is an advantage of an f.i.a. system. The measurement of the reaction can be taken at any stage. Lengthening the mixing coil, decreasing the flow-rate or stopping the flow can be employed to increase the residence time where necessary.

1.2.1.3 Components of f.i.a. system

i) Delivery system

The most frequently used delivery system is a peristaltic pump. The delivery rate can be varied by using different pump tubes and there are also pumps available which allow variation of the flow rate by different pumping speeds. There are disadvantages associated with this kind of pump: it is not completely pulse free and the pump tubes deteriorate easily causing the flow rate to alter. A displacement pump can be used instead, which requires no pump tubes.

ii) Injection system

In the early work of Ruzicka and Hansen (1975), a syringe with its needle penetrating a rubber tube was used, and was subsequently replaced by a flap valve (Betteridge 1978).
Nowadays, a rotary valve with a by-pass coil is normally used. The by-pass coil is necessary to avoid the carrier stream being stopped temporarily, interrupting its flow.

iii) Manifold and Mixing coil

The manifold is normally constructed from Teflon or polypropylene tubes which are flanged so that a standard connector can be used. In most cases the inner diameter is about 0.4 to 1.0 mm. Y or T connectors is used to converge two or more streams. The mixing coil are also constructed from teflon or polypropylene tubing. By varying length of this mixing coil the dispersion can be controlled to suit the reaction and method used.

iv) Detector

The detection system is very much dependent on the method used for analysis, from the simplest method of colourimetry to detection involving an inductively coupled plasma (ICP). To maintain the efficiency of the f.i.a. system the detection system should be fast in response and recovery.

1.2.2 Technique in f.i.a.

1.2.2.1 Stopped flow

Stopping the stream for a fixed period of time increases
the residence time of the sample allowing the reaction to get nearer to completion and avoids dispersion at the same time. The sensitivity will be increased and it may be possible to lower reagent consumption as well. In this technique the flow of the stream is stopped for a certain period of time after sample and reagent have been injected and mixed, thus the dispersion zone will stop and the dispersion will become independent of time. After this period, and the necessary reaction or incubation has taken place, the flow is restarted and the signal recorded as the mixture passes through the detector. This technique has been used by Lim et al. (1980) and Ruzicka (1979).

1.2.2.2 Intermittent pumping

This technique is primarily used to conserve reagent while increasing the washout speed. The manifold consists of two pumps which operate independently. One pump is used to propel the carrier stream and the second is used to flush the system with the wash solution (see figure 1.13).
1.2.2.3 Merging zones

The use of expensive reagent continuously, even when there is no sample present in the system, is the main disadvantage in a continuous system. The merging zone principle can be used to avoid this problem, and can be achieved in two ways: 1) by intermittent pumping or 2) by the use of multiple injection valves.

Figure 1.13 Fia manifold for an intermittent pump system.

Figure 1.14 Typical f.i.a. manifold for merging zone
In an intermittent pumping system the sample zone is injected into the flowing inert carrier solution, while the pump controlling the reagent is stopped, the pump will only be activated when the sample zone has reached the merging point, delivering reagent for a preset time then stopped again. In the merging zone technique, as suggested by Bergamin et al. (1978) and Mindegaad (1979), the sample and reagent are simultaneously injected into the two streams and travel to meet at the merging point after passing through two equal lengths of identical tubing (see figure 1.14). The drawback of this technique is that it is very difficult to obtain perfect overlapping of the reagent and sample due to the difficulty in controlling the pumping rate and the nature and geometry of tubing used. However this property may be used to an advantage by adjusting the flow-rates overlap, allowing a reading for the reagent and sample blank respectively be obtained, by taking the signal before and after the peak from the signal of the reaction mixture.

1.2.2.4 Packed column

Theoretically it has been shown that packed bed reactors — tubes packed with inert particles (glass beads) — would minimize band broadening in a flowing stream better than open tubular reactors (Tijssen 1980, Van den Berg 1980, Reijn 1981).
By filling the reactor with glass beads of 70% of the inner diameter of the tube the backpressure can be reduced and the dispersion decreased while still avoiding peak overlapping.

This principle has been applied by Anderson (1979) for the determination of nitrate and nitrite, by passing the sample through a packed column containing copper coated cadmium.

With the increasing popularity of biotechnology, the use of enzymatic determinations has also increased. Many workers have applied immobilised enzyme columns to a conventional f.i.a. system. Olsson and co-workers (1986) used a packed-bed reactor containing immobilised invertase, mutarotase and glucose oxidase to determine sucrose in the presence of glucose.

A reactor containing immobilised firefly luciferase has been used to determine ATP, creatine phosphokinase and creatine phosphate (Worsfold and Nabi 1986).

An ion-exchange minicolumn has also been applied to an f.i.a. system in the determination of anions (Faizullah and Townshend 1986)

1.2.2.5 Separation

One of the simplest methods of separation which could be
easily coupled to an f.i.a. system is dialysis. A dialysis unit can be fitted at any stage in an f.i.a. system to suit any analytical requirement.

A system including a dialysis unit with a cupro-membrane has been satisfactorily used to analyse glucose in blood samples (Hansen et al 1977).

Using a gas-permeable membrane of dimethyl silicone rubber, total carbon dioxide has been determined in plasma (Baadenhuijsen and Seuren-Jacobs 1979).

Solvent extraction, which requires the mixing and separation of two immiscible liquids is undoubtedly the most unexpected technique to be used in an f.i.a.. This technique was first demonstrated by Karlberg and Thelander (1978) in the extraction and determination of caffeine. The aqueous carrier containing the sample is interspersed alternately with very small segments of organic solvent, by the use of tee piece with a carefully position platinum side tube. Extraction takes place across the aqueous-organic interface and then the two phases are separated by a second tee piece (see figure 1.15). In this technique typical assay times are 20 to 60 seconds.

Another approach to solvent extraction was described by Bergamin et.al. (1978). The sample and reagent merge with the solvent together in a coil of tubing (0.35mm bore) and the phase then separates in a conical 0.11ml chamber.
The liquid-liquid extraction method has been successful in achieving separations and as a method of sample introduction in atomic absorption analysis (Nord and Karlberg 1983), and inductively coupled plasma (Cox and MacLeod 1986).

figure 1.15 f.i.a. manifold with solvent extraction

1.2.2.6 Reverse f.i.a.

In normal f.i.a. the solution containing the determinand is injected into an inert eluent, or into an eluent containing an excess of reagent, then the mixture determined by an appropriate detection method. In reverse f.i.a., the positions of sample and reagent solution are reversed; the reagent is injected into the sample solution which now acts as a carrier reagent. This method can be particularly
advantageous in process-control situations where
determinand is cheap and plentiful. The other advantage are
where the economics of the reagent is concerned and the
possibilities of multiple injection with different type of
reagents.
In this work, this concept is used due to scarcity and cost
of the enzyme while the sample is abundant and cheap.

1.3 Flow Injection Analysis With Chemiluminescence
Detection

This subject is discussed in chapter 2.
CHAPTER TWO
2.1 Introduction

In a typical chemiluminescence reaction, the production of light is a function of time. Its intensity can be measured at a specific time after mixing, or it can be integrated for the entire time of light emission. Since it varies with time, it is crucial that chemiluminescence reactions be initiated in a controlled manner to achieve precise measurement. A proper and reproducible mixing is normally required.

In a fast kinetic reaction such as bioluminescence reaction, much of the signal is lost while mixing is being carried out before the measurement. This will reduce the sensitivity and precision of the measurement.

Flow system has provided an opportunity for a precise, quick and reproducible mixing. The degree of mixing and the timing can be adjusted as required by the design of the manifold and the flow rate used.

In an air-segmented flow system, as employed by Scot et al. (1980), the reagent, carrier and air are pumped through, after the sample introduction, as soon as mixing occurs the reaction takes place, the light is then measured. The use
of air bubbles in this system limits the rapid reach of the reaction mixture to the detector. These air bubbles are measured together with the reaction mixture as it passes in front of the detector. The slow reach and the inclusion of air bubbles have resulted in poor precision and reproducibility.

F.i.a has an advantage over the air-segmented system in respect to these problems. The combination of chemiluminescence and f.i.a. provides extreme reproducibility of sample and reagent mixing. This is essential for precise chemiluminescence studies which is frequently lacking in static procedures. F.i.a. is also an excellent tool for measuring transient light signals produced by reactions such as chemiluminescence.

The suitability of chemiluminescence as a detection technique for flow injection has been demonstrated by Rule and Seitz (1979).

The oxidation of luminol has been the most widely used chemiluminescence reaction in the study of chemiluminescence by f.i.a. In an early attempt to combine chemiluminescence and f.i.a., Burguera and Townshend (1979) constructed an f.i.a. manifold with spectrometric flow cell placed in front of the detector. The use of cuvette type cells resulted in slow flushing and peak broadening, it was later replaced by a coiled flow cell (Burguera 1980). This design has been successfully used to determine cobalt(II)
by this reaction and also by fluorescein sensitized oxidation of sulphide by sodium hypochlorite.

The flexibility of f.i.a. has also permitted the use of an ion-exchange column. Zinc and cadmium has successfully been eluted through an ion exchange column and then passed through an f.i.a. manifold and detected by luminol-peroxide chemiluminescence (Burguera et al 1981).

This reaction has also been used to determine iron II, III and total iron by f.i.a. (Sarantonis and Townshend 1986).

Besides metal ion, microperoxidase has also been used as a catalyst in luminol-peroxide reaction (Olsson 1982) in an f.i.a. determination. F.i.a. was also coupled to HPLC for a post-column detection of amino acids. Combination of f.i.a. and liquid chromatography has also been described by MacDonald and Nieman(1985).

Combination of f.i.a. -luminol/H$_2$O$_2$/K$_3$Fe(CN)$_6$ has also been reported for an enzyme immunoassay using glucose oxidase as a labelling enzyme in determination of anti $\alpha$-ferroprotein IgG, insulin and 17-$\alpha$-hydroxyprogesterone (Maeda and Tsuji 1985).

The incorporation and use of a dialysis unit in chemiluminescence-fia system has been reported by Pilosof and Nieman(1982). While Worsfold et al(1984) has combined a dialysis unit together with a wall coated enzyme reactor in
an f.i.a. manifold for the determination of plasma glucose using luminol/H$_2$O$_2$/K$_3$Fe(CN)$_6$ luminescence.

The use of immobilized enzyme column in f.i.a. for the analysis of glucose and uric acid has also been described (Tabata et al 1984).

Another important type of chemiluminescence reaction studied using f.i.a. is that involving peroxyoxalate. Mahant et al (1983) have combined f.i.a. with chemiluminescence of bis(2,4,6-trichlorophenyl)oxalate (TCPO) in the determination of fluorescein and fluorescamine labelled species.

Bis (2,4-dinitrophenyl) oxalate chemiluminescence was used by Honda et al (1983) to determined dansylalanine using a merging zone type f.i.a. manifold with rotating mixing device at every merging point to obtain maximum mixing.

Recently Van Zoonen and co-workers (1985) has reported the use of solid form bis (2,4,6-trichlorophenyl) oxalate. A carrier of acetonitrile and water with a sensitizer, perylene, was passed through an f.i.a. manifold with incorporating a packed bed reactor containing the oxalate to determine hydrogen peroxide. The use of immobilised sensitizer has also been suggeted.

Other chemiluminescence reactions studied using f.i.a. include fluorescein sensitised oxidation of sulphide by

In bioluminescence, several methods using segmented flow system had been reported (Dufresne and Gitelman 1970; Curtis and Johnston 1979), however, the potential of the combination of bioluminescence and f.i.a. has not been fully utilised, probably due to the need for a continuous flow of expensive reagents. Grayeski et al(1977) have tried f.i.a.-bioluminescence combination and suggested several setup for ATP bioluminescence reaction. While Worsfold et al(1984) have reported the use of merging zone principle in ATP determination. Recently immobilised luciferase had also been used in an f.i.a. determination of ATP (Worsfold and Nabi 1986).
2.2 EXPERIMENTAL

2.2.1 Apparatus
An LKB 1250 Luminometer
AMFIA
ESI-PANAX HPLC Liquid Scintillation Counter
Linear Instrument chart recorder
PTFE 1.01 mm i.d. tubing as the basis for f.i.a. manifolds.
Connectors

2.2.2 Materials
ATP (Adenosine 5'-triphosphate)
Tris hydroxyaminoethane (TRIS)
ATP Monitoring Reagent
Acetic acid
Hydrochloric acid
Potassium permanganate

Note:
Commercial ATP Monitoring Reagent (LKB) is a mixture containing the following chemicals:
Firefly luciferase
D-luciferin
Bovine serum albumin 50ug
Magnesium acetate 0.5 mmol
and Inorganic pyrophosphate 0.1 umol

2.2.3 Instrumentation

Two instruments were used for bioluminescence measurements.

1) LKB 1250 Luminometer
   (LKB-Wallac, Turku, Finland.)
   LKB 1250 luminometer is basically a single photometer tube, where light from a luminescent reaction directed and focused by a concave disc is read. For calibration purpose, a radioactive source of Cl\textsubscript{3}, encased in the counting chamber, can be rotated and placed in front of the photomultiplier tube.

2) ESI-PANAX 506C HPLC Radioactivity Monitor
   (Esi-Panax, Rotheroe & Mitchell Ltd, Ruislip, Middlesex, U.K)
   This instrument is basically a liquid scintillation counter fitted with two EMI 9635Q Quartz window photomultiplier tubes giving a response of a range from 400 to 650nm.

The instrument can be operated using either one or both detectors in coincidence or non-coincidence mode. During the course of this work it has been used in non-coincidence mode.
AMFIA 2000
(American Research Prod. Corp., Bethesda, Maryland, U.S.A)
AMFIA is a complete automated instrument for flow injection analysis. It has 3 rotary pumps and a withdrawal pump. It is also equipped with a pneumatically driven valve, temperature controller and colorimeter. A single board computer, Rockwell AIM 65 was also connected to the unit. Most of this work was carried out without using the temperature controller, colorimeter and the computer unit. The computer unit was malfunctioned while the colorimeter was replaced by the luminometer described earlier.

The built-in valve unit was taken out and placed nearer to the luminometer to suit to the optimum condition for an f.i.a. setup.

2.2.4 Optimization of the Esi-Panax Parameter

Optimization of the instrumental parameter was carried out by injecting a mixture solution of ATP and ATP monitoring reagent into the manifold. When the mixture reached the detector, the flow was then stopped and the voltage window setting adjusted to obtain maximum signal output.

Zero-offset potentiometer was also adjusted to obtain a complete reduction in the noise level and give zero reading for the blank.
Instrument gain was set at X2 as recommended by the manufacturer. It was also used in non-coincidence mode with 3 second time constant unless stated otherwise.

2.2.5 The design of the f.i.a. manifold

A dual channel f.i.a. manifold has been adopted for this work. Merging zone type with dual injection valve was not possible due to unavailability of second valve in the AMFIA 2000 instrument. This manifold was adopted for several reasons. Firstly, to maximise the dispersion between sample and reagent at the T-junction. Secondly the buffer could be introduced into the flow without predilution and thirdly the possibility of recycling the unreacted ATP monitoring reagent (enzyme mixture). In a single channel f.i.a., this
would not be possible as the reagent mixed and reacted with the sample/carrier making the excess enzyme unusable. In this case the reagent would only be diluted by the buffer.

The designed manifold is shown in figure 2.1.

Teflon tubings of 1.01mm internal diameter were used all round. Exposed portions of the tubing were wrapped with black tubing and insulating tape to prevent a 'fibre-optic' effect introducing ambient light into the detectors.

2.2.6 General procedures

1) ATP Standard solution
0.152g pure ATP was dissolved in 10 ml buffer (tris/acetate) pH 7.75. The solution was then dispensed 1ml each in small dark vial and stored at temperature -18°C. Working solutions were prepared when needed by thawing these solution and serially diluted accordingly using doubly distilled water.

2) LKB Monitoring Reagent
Each vial containing lyophilised substances was diluted with 10ml doubly distilled water and kept in the freezer before use.

3) Tris Buffer
12.1V tris and 0.06 g EDTA were dissolved in 1
doubly distilled water and the pH adjusted to 7.75 using dropwise additions of either concentrated hydrochloric acid or acetic acid.

4) Cleaning
All glassware were soaked in 0.2 M HCl for several hours. It was then rinsed with distilled water, then three times with tris/HCl buffer and finally by distilled water again. For heavily contaminated glassware, Decon 90 was employed prior to acid soaking. Extra care was taken to avoid touching the tips of pipette and sample containers.
2.3 RESULTS AND DISCUSSION

2.3.1 Determination Reaction length

The reaction length is defined as the length of manifold from T-junction to the detector (A to B; see fig. 2.1). The examination was carried out using $1 \times 10^{-8}$ M ATP propelled through channel 1 and 0.1 M tris buffer (pH 7.75) in channel 2. The ATP monitoring reagent was then injected through the valve. The length of the tube was varied from 15 cm to 65 cm. Shorter than 15 cm was not possible due to the dimension of the instruments. The result obtained is shown in figure 2.2.

The optimum length was found to be about 20 cms. Comparing to other methods such as spectrometric, this length is considered short. This short length of the reaction tube is needed as the bioluminescence reactions are mostly quick and almost instantaneous. From the result at longer reaction length the reading was lower as the reaction has already passed its maximum burst of light after mixing occurred.
Figure 2.2 Determination of the reaction length.
Figure 2.3 Determination of the reagent length.
2.3.2 Determination of Reagent length

The reagent length is defined as the length between the injection valve and the T-junction in the manifold (C to A). The optimisation of this length was carried out using the same procedure as in the reaction length. The length was varied from 10cms to 65cms.

Results in figure 2.3 show that the shorter the length the higher light output obtained. A length of 15 cms was adopted due to restriction in the dimension of the detector and the injection valve.

2.3.3 Flow Rates

Channel 1: The determination was carried out by injecting 50ul ATP monitoring reagent into 1 X 10^-8 M ATP.

Figure 2.4 shows the intensity slowly increases as the flow rate increases. The maximum intensity was achieved at around 2.5 to 3.2 ml/min then it decreases slowly.

The differences in these intensities were not as large as expected. This is probably due to the use of commercial LKB enzyme preparation which gave a steady light output for some length of time, any small variation in the flow rate
Figure 2.4: Optimization of flow rate for channel 1.
Figure 2.5 Optimization of flow rate for channel 2.
would not give a large difference in the recorded intensity.

A flow rate of 2.8ml/min was adopted for this work.

Channel 2: Same procedure as in channel 1 was adopted. The flow was varied from 0.5 to 9.0 ml/min.

The efficiency of light detection was found to be better at slow flow rate (see figure 2.5). A flow rate of 1.0ml/min was adopted for this work.

2.3.4 Sample Volume

Using the PTFE tube of 1.01mm i.d. the length of the tube was varied to obtain different injection volumes.

The effect of the injection volume is shown in table 2.1 and figure 2.6. Over the range of 350 to 250ul there has not been much change in the light intensity recorded, probably due to the partial detection of the light produced in the cell tube as the length of the cell coil is shorter than the length where the mixture occupied. Also at this volume, it has been observed that there was no peak splitting as normally occur in other methods of detection. This could be the result of complete mixing at the T-junction and the light produced could well have travelled through the unreacted section of the mixture and still be detected by the photomultiplier tubes. This phenomenon has
Figure 2.6 Injection volume versus bioluminescence intensity.
also been observed in a single channel f.i.a.

<table>
<thead>
<tr>
<th>Volume (ul)</th>
<th>Peak Height</th>
<th>Ratio (Peak Ht./Vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>350</td>
<td>30</td>
<td>0.09</td>
</tr>
<tr>
<td>300</td>
<td>32</td>
<td>0.11</td>
</tr>
<tr>
<td>275</td>
<td>30</td>
<td>0.11</td>
</tr>
<tr>
<td>250</td>
<td>27</td>
<td>0.11</td>
</tr>
<tr>
<td>225</td>
<td>27</td>
<td>0.12</td>
</tr>
<tr>
<td>200</td>
<td>26</td>
<td>0.13</td>
</tr>
<tr>
<td>175</td>
<td>25</td>
<td>0.14</td>
</tr>
<tr>
<td>150</td>
<td>23</td>
<td>0.15</td>
</tr>
<tr>
<td>125</td>
<td>23</td>
<td>0.18</td>
</tr>
<tr>
<td>100</td>
<td>21</td>
<td>0.21</td>
</tr>
<tr>
<td>75</td>
<td>20</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 2.1 Effect of the volume of injection on the production of bioluminescence

For economic reasons a volume of 75ul and sometimes 50ul were adopted for this work.

2.3.5 Dispersion Coefficient

Using 0.001 M solution of potassium permanganate with a photometric detector, the dispersion coefficient was
determined by injecting permanganate solution into a flow stream of distilled water. The dispersion coefficient for this setup was found to be 2.86.

This value indicates that the setup gives a medium dispersion to the reaction mixture which is fairly appropriate for a fast reaction with good mixing requirement.

The use of a bioluminescent mixture is not possible for this determination due to its decreasing intensity with time, making maximum peak \( (C^0) \) unobtainable.

2.3.6 Standard Calibrations

Three standard calibrations were carried out using different detection methods.

1) Non-fia using LKB 1250 luminometer

The ATP standard calibration was carried out by injecting into a cuvette containing 1 ml ATP standard solution and 0.5 ml tris buffer. The mixture was mixed by a rotating mechanism and the light measured by the luminometer. Figure 2.7 shows the curve obtain for standard ATP solutions from \( 10^{-6} \) to \( 1 \times 10^{-1} \)M.

A good linearity of between \( 1 \times 10^{-10} \) to \( 1 \times 10^{-6} \)M ATP obtained on a log-log plot.
2) F.i.a. calibration using liquid scintillation in single detection mode

Using the optimised f.i.a. manifold, 75ul LKB monitoring reagent was injected to standard ATP solutions. Figure 2.8 shows curve obtained on a log-log scale with a linearity from $1 \times 10^{-10}$ to $1 \times 10^{-7}$ M ATP.

3) F.i.a. calibration using liquid scintillation counter in dual detection in non-coincidence mode

Using the procedure as in the previous determination the light was detected by both photomultiplier tube in the liquid scintillation counter.

Figure 2.9 shows a slightly non-linear curve on a log-log plot with minimum detectable limit of upto $1 \times 10^{-13}$ M ATP.
Figure 2.7 Standard curve for ATP using non-f.i.a. determination with LKB luminometer.
Figure 2.8 'Standard curve for ATP using single photomultiplier tube in a liquid scintillation counter.
Figure 2.9 Standard curve for ATP using liquid scintillation counter in non-coincidence mode.
2.4 CONCLUSION

The detection of ATP by f.i.a. is simple and convenient compared to the conventional batch method. The task of selecting the varying signals and the tight timing required for each analysis are easily eliminated.

The design of f.i.a. manifold is also very simple. It does not need a long reaction coil, in this case only a 20 cms tube was required. Fast flow rate can also be employed in this determination as the reactions are normally fast and spontaneous. With the optimised design a sampling rate of upto 120 samples per hour can be easily obtained.

The detection limit and linearity are comparable to the conventional system, it could have been better if only the detector was functioning properly during the course of this work. A better and more stable instrumentation could probably improve the results.
CHAPTER THREE
3.1 INTRODUCTION

3.1.1 Product Inhibition

The firefly bioluminescence reaction proceeds according to the following equations:

\[ E + LH_2 + ATP \rightarrow E.LH_2AMP + PPI \]
\[ E.LH_2AMP + O_2 \rightarrow E + CO_2 + AMP + oxyluciferin + light \]

Besides the formation of oxyluciferin, carbon dioxide, AMP, pyrophosphate and light, luciferase will also catalyse the formation of dehydroxyluciferyl adenylate (L-AMP) as shown in the reaction below:

\[ E + L + Mg.ATP \rightarrow E.L-AMP + PPI \]

This property has been reported by Green and McElroy (1956) and DeLuca (1976). The product (L-AMP) is a potent inhibitor of bioluminescence and a competitor with luciferin for the active sites on the enzyme luciferase resulting in less available luciferase for the production of the light. The production of this light can however be slightly restored by the addition of inorganic pyrophosphate. The continuing production of the pyrophosphate by the same reaction will create an excess which will in turn inhibit the bioluminescence process as a
high concentration of pyrophosphate and sulphate ions inhibit the action of the luciferase enzyme (DeLuca et al., 1964).

AMP, another product of the reaction has also been reported to interfere in the bioluminescence process, especially when some lytic enzymes are present in the reaction mixture. During the process, the AMP will be converted to ADP and eventually to ATP which will subsequently interfere in the actual determination by giving an artificially higher signal reading.

The conversion reactions of the AMP and ADP have been used to detect the total adenylate content of ale yeast (Hysert et al., 1977).

3.1.2 Matrix effect

i) Urine Matrix

The determination of ATP in urine has been used as a basis of bacteriuria screening (Thore et al. 1975; Alexander et al. 1976). Urine normally contains various substances which inhibit bioluminescence (Conn et al. 1975). These species will apparently reduce the production of light. Several substances have been mentioned as source of interference such as chloride (Denburg and McElroy 1970) and sulphate (DeLuca 1979). Nichol (1978) has shown that by diluting the urine before analysis, the inhibition can be reduced.
drastically.

ii) Surfactants
Recent study by Kricka and DeLuca (1982) found that the activity of the enzyme luciferase increased in a matrix of non-ionic surfactants. This phenomenon had not been observed in cationic and anionic surfactants.

iii) Buffer
As mentioned in chapter 1 the optimum pH for ATP reaction is about 7.75 giving peak maximum emission at 560nm. A change in pH will result in the change in peak maximum thus the measured intensity. The production of light will also be affected by the type of buffer used. The interferences from buffers are mainly from the ionic species in the buffer salt used. Gilles et al. (1976) compared several buffers and found that a tris-maleate buffer gave the least inhibition while the phosphate gave about 80% inhibition.

3.1.3 Contaminating Enzymes

Although the luciferin-luciferase reaction is specific for ATP, other nucleotides are also known to react with the crude enzyme. This is primarily due to the presence of nucleotide diphosphate kinase and adenylate kinase (Rasmussen 1968). Among other nucleotides, GTP has been pointed as the most probable contaminant. In this case, the use of a slow integration time has been suggested.
In an attempt to avoid interference from these enzymes, Karl and Holm-Hansen (1978) suggested the use of GDP on the enzyme prior to ATP determination, which would cause the inhibition of ATP produced from other nucleotides.

There are various other enzymes which would interfere in the detection of the ATP. The release of ATPase and pyrophosphatase during the extraction of bacterial ATP has also been reported (Abrams 1965). These substances can cause problems during the detection of ATP. The use of a high temperature extraction method e.g. boiling tris has an advantage as these enzymes are denatured by the heat.

In recent years the problem has been solved by purifying the crude enzyme used in the determination. Several methods of purification have been suggested such as isoelectric focusing (Lundin & Myhrman 1971), affinity chromatography (Branchini et al. 1980) and gel filtration (Nielsen & Rassmussen 1968 and Momsen 1977). The elimination of these enzymes has resulted in an increased intensity of light produced and a greater stability of that light.
3.1.4 Solvent

Organic solvents are known to alter the ionic form of luciferin by shifting the wavelength of the peak maximum of the emission spectrum. The use of ethanol, isopropanol, butanol and chloroform for the extraction of bacterial ATP from microorganisms will leave traces of these organic substances. The effects of these solvents were investigated in this work.

3.1.5 Ionic Inteferences

Ionic species are naturally present in the solution, normally introduced by either the addition of buffer or an extracting agent.

Gilles et al (1976) showed that inhibition of anions in the order of acetate $< \text{Cl}^- < \text{I}^- < \text{ClO}_4^-$
3.2 EXPERIMENTAL

3.2.1 Material

ATP (Adenosine 5-triphosphate)
LKB Monitoring Reagent
Tris (Tris hydroxyaminoethane)
ATPase
Pyrophosphatase
sodium phosphate
sodium pyrophosphate
Methanol
Ethanol
Diethyl ether
Chloroform
Methyl isobutyl ketone (MIBK)
Acetone
Ringer solution
3.2.2 General Procedure

ATP, LKB monitoring reagent and Tris buffer were prepared as described in section 3.2.2

Apyrase - a vial containing ATPase(E.C.3.6.1.3) and ADPase (100 units) was diluted to 2 ml with doubly distilled water. The solution was then dispensed according to experimental requirements.

Inorganic pyrophosphatase(pyrophosphate phosphohydrolase E.C 3.6.1.1) - 1 ml doubly distilled water was added to a vial containing 100 units of lyophilized pyrophosphatase. The solution was then dispensed accordingly.
3.2.3 Methods

3.2.3.1 The Effect of Immiscible Organic Solvents on ATP

Water (20ml) was shaken with some organic solvent for about 20 minutes and then left to saturate. A volume of 10ml of the aqueous layer was then pipetted into a sample tube and 1ml of $10^{-7}$ M ATP added. The bioluminescence was then detected. The excess sample was then immersed into boiling water bath for another 10 minutes and then bioluminescence detected.

3.2.3.2 Effect of Miscible Organic Solvent

The effects of miscible organic solvents were carried out using three different solvents. Each of the solvent was added to solution of ATP($1 \times 10^{-8}$) and the bioluminescence was then detected. Further test were carried out by adding the solvents to a serially diluted ATP solution and the bioluminescence detected.

3.2.3.3 Interference By ATPase

This investigation was carried out by the addition of ATPase(200ul) to 10 ml $1 \times 10^{-8}$M ATP solution. The ATP bioluminescence was then determined.
Elimination of ATPase was carried out by reacting Propranolol (0.1ml, 0.4mg/ml) and magnesium (0.1mg/ml) with ATP solution containing ATPase. After incubation the bioluminescence was detected.

3.2.3.4 Interference of Pyrophosphate on ATP Bioluminescence

The experiment for the effect of pyrophosphate and phosphate were carried out by the addition of these compounds to ATP solution (1X10^{-8} M) the bioluminescence was then detected.

In the elimination of pyrophosphate, solution of ATP was added with pyrophosphatase enzyme. The solution was left for few minutes and the bioluminescence detected.

The effect of pyrophosphatase was studied by adding pyrophosphatase (x ul solution of 100 units in 10 ml water) to solution of ATP (1 X 10^{-8} M) containing sodium pyrophosphate (50 ul 2.2g/l).

3.2.3.5 Ringer Solution

The effect of Ringer solution was studied by adding the appropriate amount of each of the studied substances as described in the preparation of the Ringer solution to ATP solution (1 X 10^{-8}).
3.3 RESULTS AND DISCUSSION

3.3.1 The Effect of Immiscible Organic Solvents on ATP

This investigation was carried out to establish the effect of water immiscible organic solvents on the ATP bioluminescence.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Intensity (10^5)</th>
<th>Intensity(10^5) after boiling</th>
<th>boiling point/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>53.50</td>
<td>49.0</td>
<td>100</td>
</tr>
<tr>
<td>CCl</td>
<td>6.15</td>
<td>5.0</td>
<td>76.75</td>
</tr>
<tr>
<td>Dichloro methane</td>
<td>4.5</td>
<td>20.0</td>
<td>40.00</td>
</tr>
<tr>
<td>Diisopropyl alcohol</td>
<td>1.5</td>
<td>0.0</td>
<td>83.00</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.5</td>
<td>18.0</td>
<td>61.20</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>2</td>
<td>18.0</td>
<td>40.00</td>
</tr>
<tr>
<td>water *</td>
<td>38.5</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>MIBK *</td>
<td>7.5</td>
<td>24.0</td>
<td>114</td>
</tr>
</tbody>
</table>

* (1 X 10^-8 M ATP using an LKB 1250 luminometer)

Table 3.1 The effect of water saturated organic solvent on the production of light by ATP bioluminescence.

The results in table 3.1 show that all of the organic solvents tested inhibit the bioluminescence reaction. However, with the exception of carbon tetrachloride and
diisopropyl ether, the light production was partly restored after the solution was boiled. This behaviour indicates that the ATP, luciferin and luciferase were not affected or react with these organic solvents. The reduction in the signal was probably due to the blue shift in the emission spectrum (see chapter 1). After these solvents were boiled off the peak maximum was restored back to the red region. The is due to the formation of different ionic form of luciferin in aqueous and organic solvents (see chapter 1).

Carbon tetrachloride and diisopropyl ether have high boiling points compared to the other solvents. These solvents stayed in the mixture even after boiling causing the inhibition of the bioluminescence reaction.

3.3.2 Effect of Miscible Organic Solvent

Ethanol and methanol were used by StJohn (1970) while Chappelle and Levin (1977) used acetone to extract ATP from bacteria and yeast. In their methods these solvent were decanted after the extraction and the excess boiled off. In this work the solvent used were tested to see the effects of these solvents on bioluminescence.

i) methanol

Figure 3.1 shows the effect of methanol (1ml) on a serially diluted ATP solution. The intensity decreased as the volume of methanol added increased (figure 3.2). At about 2:3 ratio of water : methanol the production of light is
Figure 3.1 Standard curve for ATP in water and methanol.
Figure 3.2 The effect of methanol on ATP bioluminescence.
completely inhibited. The addition of a smaller volume of methanol figure 3.2 caused the quenching of the intensity by about 50%. On addition of 0.005ml methanol to a 10ml sample the bioluminescence was still reduced by about 50%, this indicates that the presence of methanol, even at very small volumes quenches the light production as oppose to the suggestion by Knust et al (1975).

ii) ethanol
Ethanol does not have any effect on the production of light by ATP reaction. The results in table 3.2 show that in an ethanolic matrix, there was no inhibition demonstrated by ethanol present except for some variation due to experimental error which was probably caused by the mixing between ethanol and the aqueous ATP solution. A variation of about 1 to 30% was observed.

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Bioluminescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38.17</td>
</tr>
<tr>
<td>10</td>
<td>27.17</td>
</tr>
<tr>
<td>5</td>
<td>39.50</td>
</tr>
<tr>
<td>1</td>
<td>31.00</td>
</tr>
<tr>
<td>0.5</td>
<td>39.17</td>
</tr>
<tr>
<td>0.1</td>
<td>38.00</td>
</tr>
<tr>
<td>0.01</td>
<td>32.00</td>
</tr>
<tr>
<td>0.001</td>
<td>30.67</td>
</tr>
</tbody>
</table>

Table 3.2 The effect of ethanol solvent on the light production
Figure 3.3 Standard curve for ATP in water and acetone.
Figure 3.4 The effect of acetone on ATP bioluminescence.
The results indicate that ethanol does not cause any inhibition to the reaction as expected. As ethanol has a slightly higher ionizing capability than methanol, luciferin might still be present in the dianion form producing light production as in water.

iii) Acetone

The effect of acetone was also investigated as it had been used to extract ATP from E.Coli (Chappelle and Levin 1968 ). This investigation shows that acetone quenches the bioluminescence reaction. Figure 3.3 shows the effect of acetone (0.5ml) on standard ATP solutions.

The intensity of the light produced decreased when volume of acetone in the matrix increased as shown in figure 3.4. A complete reduction in the signal produced was observed when the acetone concentration reached 1%. These results suggest that the presence of acetone inhibit the bioluminescence reaction.

The boiling off process was also studied to monitor the recovery of the light production. The results indicate that the light intensity was restored to about 80-90%, due to volatility of acetone, the solutions tend to boil and spill over making the result very unreliable. A more gentle heat treatment is needed and great care should be taken when acetone is used in the extraction of bacterial ATP.
Figure 3.5 The hydrolysis of ATP by ATPase.
All of these solvents investigated above did not give any significant blank reading.

3.3.3 Intereference By ATPase

The inhibition of ATPase can be described as linear to the presence of ATP in the solution as shown in figure 3.5. With the addition of 20ul of 200 units to a 10 ml $1 \times 10^{-8}$ M ATP the light production was reduced by about 35%. Addition of 0.1 ml ATPase gave a complete reduction in the light produced.

Method employed by Abazankuwe (1986) was adapted in an attempt to eliminate the ATPase interference in an experiment. Propranolol (0.1ml, 0.4mg/ml) and magnesium (0.1mg/ml) were added to ATP solution containing ATPase. Result failed to show any restoration of light produced. Test also found that propranolol quenches the bioluminescence reaction.

3.3.4 Intereference of Pyrophosphate on ATP Bioluminescence

Figure 3.6 shows the result when sodium pyrophosphate solution was added to the ATP reaction.

From these results it can clearly be seen that pyrophosphate strongly inhibits the bioluminescent reaction. Using phosphate (200ul, $0.001$ M) and pyrophosphate
Figure 3.6 The effect of inorganic pyrophosphate on ATP bioluminescence.
Figure 3.7 The effects of inorganic pyrophosphate and phosphate on ATP bioluminescence.
Figure 3.8 The effects of pyrophosphatase enzyme on ATP bioluminescence.
(200ul, 0.001M) as interferences in the ATP bioluminescence reaction showed that the pyrophosphate is a stronger inhibitor of the reaction than phosphate (figure 3.7).

The possibility of using an enzyme pyrophosphatase was also investigated. However, investigation shows that pyrophosphatase also inhibit bioluminescence reaction as shown in figure 3.8. This effect has also been reported by DeLuca (1975).

The results presented in table 3.3 show that the pyrophosphatase is not effective at restoring the production of light. The amount of pyrophosphatase is clearly not adequate for the amount pyrophosphate present in the solution. Due to economic and practical reasons, the use of larger volume of pyrophosphatase has not been made possible.

<table>
<thead>
<tr>
<th>volume (ul) pyrophosphatase</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>69.5</td>
</tr>
<tr>
<td>10</td>
<td>60.5</td>
</tr>
<tr>
<td>20</td>
<td>58.0</td>
</tr>
<tr>
<td>30</td>
<td>64.0</td>
</tr>
<tr>
<td>40</td>
<td>66.5</td>
</tr>
<tr>
<td>50</td>
<td>60.5</td>
</tr>
</tbody>
</table>

Table 3.3 The effect of pyrophosphatase on the restoration of ATP bioluminescence.
This investigation shows that the pyrophosphate, phosphate and pyrophosphatase all inhibit the ATP bioluminescence reaction. The use of pyrophosphatase to eliminate the pyrophosphate produced in the solution is also shown to be ineffective as the enzyme itself inhibits the bioluminescence reaction.

3.3.5 Ringer Solution

<table>
<thead>
<tr>
<th>Material</th>
<th>Bioluminescence intensity (X 10^5 counts/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>26.17</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>25.50</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>38.83</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>4.50</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>11.17</td>
</tr>
<tr>
<td>Ringer's solution (full strength)</td>
<td>12.33</td>
</tr>
<tr>
<td>Ringers solution (1/10 strength)</td>
<td>28.00</td>
</tr>
</tbody>
</table>

Table 3.4 The effect of Ringer's solution and its constituents on ATP bioluminescence.

Ringer's solution was used in all dilutions and the suspensions of bacteria throughout this work. The effect of the Ringer and its constituents on the bioluminescence reaction were examined. Table 3.4 shows the effect of the individual substances on ATP reaction. The Ringer's was prepared according to the method described in Appendix III. The reaction was carried out by adding 1ml of each of the
Figure 3.9 The effect of sodium chloride on ATP bioluminescence.
items to $1 \times 10^{-8}$ M ATP. The bioluminescence was determined by f.i.a. using a liquid scintillation counter.

The results in the table show that the full strength Ringer solution strongly quenches the ATP bioluminescence by depriving the active site of the luciferase to ATP access. However at lower concentration, hence lower ionic strength, the bioluminescence was fully restored, as the ionic strength becomes less the active site becomes available again.

Examining each of the Ringer solution constituents, sodium chloride showed the strongest inhibition, it also has the highest concentration in the solution. The effect of NaCl on ATP is shown in figure 3.9.

Sodium hydrogen carbonate enhances the ATP reaction. This is probably due to the increase in the pH value of the solution resulting in a better light signal compared to at lower pH. Results in table 3.5 show the effect of adding 0.1 ml NaHCO$_3$ to $1 \times 10^{-8}$ M ATP. However further addition of NaHCO$_3$ would result in a high ionic strength which would then affect the activity of the luciferase as in the cases described previously.
<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>Bl intensity (X 10^5 cps)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40.00</td>
<td>7.75</td>
</tr>
<tr>
<td>0.0005</td>
<td>47.17</td>
<td>8.37</td>
</tr>
<tr>
<td>0.001</td>
<td>46.33</td>
<td>8.20</td>
</tr>
<tr>
<td>0.005</td>
<td>31.67</td>
<td>8.16</td>
</tr>
<tr>
<td>0.01</td>
<td>22.00</td>
<td>7.98</td>
</tr>
<tr>
<td>0.05</td>
<td>17.83</td>
<td>7.29</td>
</tr>
<tr>
<td>0.1</td>
<td>12.17</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Table 3.5 The effect of NaHCO₃ on ATP bioluminescence.

The results showed that diluted Ringers solution (10 X) did not inhibit the reaction. This phenomenon had been taken into consideration whenever Ringers solution was used in the bacterial suspensions.
3.4 CONCLUSION

Chloroform, carbon tetrachloride and dichloromethane were found to interfere in the determination of ATP even in small volumes. The need for a vacuum extraction and evaporation after the extraction of bacterial ATP when using these solvents, clearly makes this method unsuitable for use in an on-line continuous flow method.

The use of methanol and acetone as reported (StJohn 1970 and Chappelle and Levin 1968) in the ATP extraction procedure might also be unsuitable as these solvents interfere with the determination of ATP. Work carried out showed strong inhibition by these solvents on bioluminescence. Ethanol was the only solvent which did not inhibit the bioluminescence reaction.

ATPase, an ATP hydrolysing enzyme, may cause severe interference if it is present in the solution being determined. Investigation showed a linear relationship between the amount of ATPase and the amount of ATP present in solution. The release of ATPase from the bacterial cell during the extraction of ATP may also cause interference. The great advantage of using extraction methods involving heat is that most of the enzymes present in the solution including ATPase, will be denatured by the heat thus reducing interference.
Pyrophosphate, a product in the ATP-luciferin/luciferase reaction, showed strong inhibition on the bioluminescence reaction. The use of the enzyme pyrophosphatase, to hydrolyse the interfering pyrophosphate has been shown to be inadequate. The quenching has only been transferred from pyrophosphate to phosphate. The cost and required amount of this enzyme has made uneconomical to be used in larger quantities. The only solution to this problem is to avoid steady state measurement as suggested by Lundin and Thore (1975). The use of flow system, such as f.i.a., is an advantage as the reaction is monitored as soon as the reaction takes place and does not require the steady state to be achieved.
CHAPTER FOUR
4.1 INTRODUCTION

4.1.1 Enumeration of Bacteria

Quick and reliable quantitative estimates of bacteria and biomass are rarely achieved. An ideal method of counting would allow a microbiologist to count the organisms present and estimates the proportion which is viable and metabolically active.

The presently available methods fall into two categories namely viable count and total count.

i) Viable count

Viable count methods rely on the production of visible growth of bacteria, which usually takes a few hours to not several days to obtain results. The methods are not a hundred percent accurate, always the condition in which the organism is grown does not represent the condition in which it would be in a real sample. However, this method has been accepted as an assessment, and estimates of the numbers of active bacteria.
Viable count may be divided into two broad categories a) colony count, b) Most Probable Number.

Colony count
In a normal plate count, agar nutrient is poured into a petri dish, then the bacterial aliquot of a suitable dilution is dropped and spread. The dish is then incubated for a certain period of time for the organism to divide and grow. After that period, spots of colonies are visible and counted accordingly. Jones (1970) and Postgate (1969) have suggested that for reliable results a count of 40 to 60 colonies per plate should be taken.

From this conventional method Miles and Misra (1938) suggested the drop plate count method, where a series of drops of a suitable dilution are spotted on a nutrient agar and allowed to dry and then incubated. This method is simple but less accurate than the conventional method.

Membrane filtration is another method used in viable counting. In this method, a volume of sample is filtered through a membrane which is then incubated on an absorbent pad, usually of glass fibre or cellulose, soaked in a liquid medium. Counting can be carried out either by microscope at an early stage, or using visible spots after incubation (Green et al 1975, and Lin 1976).

For percentage viability studies, Postgate (1969) suggested the use of agar medium in a steel or plastic rig on a glass
slide, the sample is inoculated with a loop and covered by a glass slip. After 2 or 3 divisions, the bacteria are counted using a microscope.

Most Probable Number (MPN)

Halvorson and Zigler (1933) suggested the use of a series of dilution of sample inoculated into or on growth medium for each dilution. After incubation each growth is recorded. The degree of dilution and the number of growth at different dilutions are then compared with values from a table to obtain MPN values. This method usually uses liquid medium in test tubes for indicator bacteria with tenfold dilution and five replicates for each dilution.

ii) Direct Counting

For cell counting, a microscope is normally used with a counting chamber. A counting chamber is a glass slide which is ground down to known depth. The ground section includes a ruled graticule area surrounded by a moat where excess fluid is run down. A drop of sample is put on the ruled area and a cover slip is then placed over it. The organisms are then counted, and the number per unit volume obtained, sometimes after staining to help in the counting.

There are several types of chambers in use, such as Fuch-Rosenthal, Petroff-Hanser and Helber Chamber. The advantages and disadvantages of these chambers have been
discussed by Mallette (1969).

This method of counting has been used extensively to obtain a total count. The presence of other particles and motile bacteria will produce a large error in the counting.

4.1.2 Other Methods

i) ATP Bioluminescence

ATP which is essential to all living cells and present in a fairly constant proportion, can be used as cell index. The reaction of ATP and luciferin in the presence of oxygen, magnesium and an enzyme luciferase will result in the emission light. This reaction can be analytically applied to enumerate bacteria and biomass. A more detailed discussion is described in chapter 1.

ii) DNA (deoxyribonucleic acid)

DNA is also present in all living cells, but unlike the ATP, it remains intact for sometime after cell death. After extracting DNA using acetone and trichloro acetic acid, DNA is then reacted with 3,5-diamino-benzoic acid dihydrochloride to form a fluorescent complex. The complex formed is then measured spectrofluorimetrically using 420nm as the excitation wavelength and 520nm as the emission wavelength. (Kissame and Robius 1958 and Holm-Hansen et al 1968).
iii) Muramic acid

N-acetylmuramic acid which is contained in the cell wall has also been used to obtain biomass index. Moriarty (1977) has converted muramic acid to NADH by various enzymes, the NADH is then assayed by bacterial luciferase.

King and White (1968), after chromatographic separation, reacted the separated muramic acid with CuSO₄ and p-hydroxydiphenyl in ether and the coloured material read at 560nm.

iv) Luminol chemiluminescence

The catalysis of the chemiluminescence of luminol-peroxide reaction by iron phorphyrin which is present in the bacteria has been used by Oliencaczi et al (1968) as a basis of the bacteria enumeration method. A problem associated with this method is the inselectivity of the luminol-peroxide chemiluminescence reaction as the presence of other metal ions will produce false results.
4.1.2 Bacterial Enumeration By ATP Bioluminescence

The Extraction of Bacterial ATP

In the method of bacteria enumeration by ATP bioluminescence, the ATP has to be extracted first before the reaction can take place. Principally this can be carried out by either extracting the intracellular contents through the cell membrane or by breaking up the cell wall completely.

The cell wall of a bacterium is a structure built to protect and support the bacterium itself. The cell wall is built of a large polymeric structure called peptidoglycan (figure 4.1).

The peptidoglycan is composed of glycan strands cross-linked by short peptides, the strands consist of repeating units of 3-1,4-N-acetylglucosamine-3-1,4-N-acetylmuramic acid. In gram positive bacteria, the cell wall contains about 15 to 50 layers of peptidoglycan. While in gram negative bacteria, it is thinner than that of gram positive bacteria with only about 3 to 8 layers situated in the zone between the cytoplasmic and the outer membrane.

The bacterial cell wall can be disrupted in several ways. The use of ultrasound with frequency of about 20 to 40kHz
Figure 4.1 Diagramatic representation of a typical bacterial cell. (Freeman 1979)

cy=cytoplasm
cm=cell membrane
cw=cell wall of gram-positive bacteria
om=outer membrane of gram-negative bacteria
pg=peptidoglycan layer
ps=periplasmic space
causes 95% disruption of the cell walls in about 5 minutes (Freeman 1979) have shown that ATP can be extracted by this method.

For a complete destruction, Imshenetskii and co-workers (1977) have used a frequency of 1.6MHz to extract ATP from sarcina flava. The optimum time for the extraction is very much dependent on the number of cells in the suspension. They have also found that for longer periods of more than 20 minutes the latent ATPases activate and hydrolyses the ATP present, resulting in less ATP extracted.

One of the most widely used methods of extraction is by cell dialysis. The cytoplasm can be extracted by boiling in an alkaline solution(Takano et al 1965). Wettermark and Stymne (1975) extracted ATP from paramecium and peridinium cells using sodium hydroxide and sodium chloride. The most successful method reported requires the cells to be boiled with tris buffer at pH 7.75. This can be carried out for either gram positive or gram negative bacteria. The use of several combinations of tris buffers such as tris acetate and Tris/EDTA (Lundin and Thore 1979) have also been reported.

Phosphate buffer has also been used (Takano et al 1965), but has the drawback of having an inhibition effect upon the action of luciferase in the determination of ATP.

Though the lysis is supposed to take place in an alkaline
solution, Bagnara and Finch (1972) have shown that boiling in water can also result in bacterial lysis to a certain extent. They have demonstrated that although this method might give a high extraction of ATP, the subsequent hydrolysis of the ATP in hot water normally results in the apparent ATP being too low.

Detergents or surfactants have been widely used as antiseptics and bactericides. A detergent can destroy the interaction between a membrane protein and a lipid. Since the bacterial surface is normally negatively charged with respect to the surfactant, cationic surfactants are normally more effective due to the attraction of different charges. Certain detergents have been shown to solubilise the cell wall (Smith 1977). In determination of ATP, Triton-X 100 had been used to lyse non-bacterial cells which interfere in the determination of bacterial ATP.

Acid denaturation of the proteins in the cell wall can also be used as the basis of an ATP extraction method. The use of perchloric acid and trichloroacetic acid have been described (Myhrman et al 1978) while Anshen (1975) used sulphuric acid in an extraction procedure which was then neutralised by potassium hydroxide before the ATP determination.

Coon et al (1975) has commented on the use of perchloric acid as an extracting agent as it causes inhibition of the luciferase reaction, while Guinn and Eidenbock (1972) used
ether to remove the acids used in the extraction procedure to avoid their interference in the ATP determination.

The use of formic, acetic and nitric acids (Knust et al 1975) have also been reported, giving different degree of extraction. The use of acids for extraction of bacterial ATP is not favoured due to inhibition by the anions of these acids, and also the need for pH adjustment resulting in the lowering of the limit of detection. In a flow system, this would require another stream for the buffer giving a more complicated manifold design.

One of the most efficient methods of cell lysis is enzymatic as the peptidoglycans are very susceptible to the hydrolytic activities of enzymes. Lysozyme (N-acetyl-muramidase) hydrolyses the glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in the glycan strand. The enzyme depolymerises the peptidoglycan which leads to the breakdown of cell wall, releasing the ATP. Repaske (1956) demonstrated the lytic property of lysozyme.
4.2 EXPERIMENTAL

4.2.1 Material and General Methods

ATP
ATP Monitoring Reagent
Picoex B
Lysozyme
ATP Monitoring reagent
Polyethylene glycol
Triton X-100
Tween-80
Brij 35
DBSA (Dodecylbenzenesulphonic acid sodium salt)
Mannitol
BDHA (Benzyldimethyl-n-hexadecyl ammonium chloride)
Cetrimide
Cetyl pyridinium bromide
Tris buffer
EDTA

4.2.2 General Procedures

1) ATP and ATP monitoring reagent were all prepared as described by the manufacturer as described in section 2.2.6.
2) Picoex B a vial containing lyophilised substance was diluted to either 2 or 5 according to the requirement. Manufacturer recommended volume is 2ml.

3) Surfactant - 1% each of the surfactant were prepared and it was then diluted to 0.001% and 0.1ml used for each solution.

4) Lysozyme (mucopeptide N-acetylmuramoyl hydrolase EC 3.2.1.17) - lysozyme solution was prepared from a commercial grade 1 derived from egg white, lyophilised powder by diluting with doubly distilled water

5) Microbiological sample

Escherichia Coli was used in all the investigations.

Preparation of sample:- Using a wire loop, bacteria were inoculated into a nutrient broth and incubated overnight. The bacteria was then centrifuge and washed with quarter strength Ringer solution. The washing and centrifuge was carried out twice to obtain clean and pure sample.

Dilution of sample:- Dilution of bacterial sample were carried out using either sterilised Ringer solution or Tris buffer in clean sterilised sample tube.

Handling of the sample:- Due to agility of the bacterial sample and the sensitivity of the ATP-Bi method, great care
has been taken to avoid contamination with foreign ATP. The bacterial suspension was also kept in the fridge to minimise its activity during storage. Experiments were carried out at minimum possible delay to avoid regeneration and destruction of the bacteria.

Standard Enumeration:— Two methods of enumeration were carried out:
1) Plate count- suspension of bacteria was spread on a agar-nutrient plate and incubated at 37°C for 17 hours.
2) Microscopic count Counting was carried out using an improved Nabaeur counting chamber using a suitable dilution.

4.2.3 Methods

4.2.3.1 The extraction of bacterial ATP using ultrasound.

The extraction of ATP from the bacteria was carried out by subjecting the organisms to an ultrasonic environment. The original f.i.a. manifold (figure 2.1) was modified to include a coil immersed in an ultrasonic bath (see figure 4.2). The extracted ATP was then mixed with the ATP monitoring reagent from channel 2 and the light produced detected by a liquid scintillation counter.
4.2.2.2 The extraction of bacterial ATP using lysozyme.

As in the previous method the original f.i.a. manifold was modified to accommodate heat treatment required for the extraction in this method. The modified manifold is shown in figure 4.3.
A third channel with flow rate of 1.0ml/min was included to propel the lysozyme solution. Two coils were also included with the first immersed in a hot water bath while the second in a cold water bath. The extracted ATP was then determined using the same procedure as in standard solution of ATP.

4.2.2.3 The investigation of bacterial ATP extraction using various surfactants.

The extraction was carried out using various surfactants by adding the surfactant (1ml 1%) to a bacterial suspension (10ml). The mixture was then left for about 10 minutes and then the extracted ATP determined using method described in section 2.2.5.
4.2.2.4 The extraction of bacterial ATP using nitric acid.

A procedure by Knust et al (1975) had been adopted. To an E.Coli suspension (10ml), nitric acid (1ml 0.2N) was added and left for about 10 minutes, it was then diluted with water or with tris buffer (10ml) for pH adjusted experiment. The extracted ATP was then determined by f.i.a.

Standard curve was constructed by adding Picoex B (200ul, 5ml dilution) to a serially diluted bacteria suspension (10ml). The ATP was then determined using f.i.a. described in section 2.2.5.

4.2.2.5 The extraction of bacterial ATP using Picoex B.

In a non-f.i.a. determination using Picoex B extraction, method described by Sanville (1976) was adopted. Picoex B (50ul, 2ml dilution) was added to a bacteria suspension (200ul) in a sample cuvette. ATP monitoring reagent was then injected into the mixture and the mixing initiated by a mechanical device. The light was then detected using LKB 1250 luminometer.

F.i.a. determination was carried out by adding Picoex B (100ul, 5ml dilution) to a bacteria suspension. The mixture was then incubated for 10 minutes and the ATP determined as in the previous experiment.
4.3 RESULTS AND DISCUSSION

4.3.1 Ultrasonic Extraction

In this part of the work, two types of ultrasonic generator were used, i.e. cell disintegrator and ultrasonic bath.

The length of the coil determined the residence time of the bacterial solution in the ultrasound environment. Experiment was carried out to find out the optimum residence time by varying the length of the coil in the ultrasonic bath. Figure 4.4 shows the optimum coil length of between 200 to 250cms which would give about 6 sec residence time. A length of shorter than this gives lower extraction, probably due to less exposure hence less ATP being released. While longer than 250cms the prolonged treatment might kill some of the organisms and reducing the availability of the intracellular ATP in the sample solution. Prolonged exposure to ultrasonic would activate hydrolyase and ATPase in the solution, thus reducing the amount of detectable ATP (Imshenetskii et al 1977). Results obtained in this experiment was clearly in agreement with this finding. The optimum coil length of 200cms was selected for use in the subsequent experiments using ultrasound.
Figure 4.4 The determination of extraction coil length in the ultrasound.
Figure 4.5 The effect of ultrasound intensity on the extraction of bacterial ATP.
The intensity of ultrasound used in the extraction procedure was also studied. Since the ultrasonic bath used was not equipped with intensity controller, a cell disintegrator was used instead. Result in figure 4.5 shows the effect on the efficiency of the bacterial ATP extraction at various intensity level of ultrasound. As expected the extraction increased as the intensity increased, although at very low intensity the extraction was not observed. This could be due to initial process of decoagulation of bacterial aggregates.

Using the obtained figure for coil length and the amplitude of the ultrasound a standard curve for E.Coli was constructed. Figure 4.6 shows the curve obtained from ultrasonic bath treatment while figure 4.7 shows the curve obtained when using cell disintegrator.
Figure 4.6 The extraction of bacterial ATP using ultrasonic bath.
Figure 4.7 The extraction of bacterial ATP using a cell disintegrator.
4.3.2 Extraction of Bacterial ATP by Lysozyme

The breaking of cell walls of bacteria using lysozyme and EDTA has been reported by Repaske (1956). In an attempt to extract the bacterial ATP, preliminary study was carried out to adapt the method as used to f.i.a. Experiments were carried out using addition of EDTA and then followed by lysozyme. Results in table 4.1 show no extraction when EDTA was added. Further test showed inhibition by EDTA. It was concluded that there were two possible explanation, firstly the EDTA had not effectively weaken the cell wall for possible attack by lysozyme, and secondly the EDTA itself was found to supressed the production of light as it complexes with the available magnesium in the solution.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bioluminescence Intensity ( X 10^3 counts/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>20.00</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>131.50</td>
</tr>
<tr>
<td>EDTA (0.001M)</td>
<td>38.00</td>
</tr>
<tr>
<td>Lysozyme + EDTA</td>
<td>108.00</td>
</tr>
</tbody>
</table>

Table 4.1 Preliminary study of the extraction of ATP using lysozyme and EDTA

Further experiments were carried out using f.i.a. manifold as described in section 2.2.5 replacing EDTA with a heat treatment.
Figure 4.3 The effect of temperature on extraction of bacterial ATP using lysozyme.
Table 4.2 shows the effect of lysozyme and the heat using 10ml E.Coli suspension and 1ml lysozyme solution. The extraction of ATP by lysozyme with heat treatment was found to be quite considerable compared to extraction by lysozyme alone. However this extraction was not as efficient as the extraction using boiling tris as described by ASTM (American Society for Testing and Materials Standards).

<table>
<thead>
<tr>
<th>Test</th>
<th>BL Intensity (x10^3 cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>6</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>176.5</td>
</tr>
<tr>
<td>Lysozyme + heat(84°C)</td>
<td>30.0</td>
</tr>
<tr>
<td>Boiling Tris</td>
<td>offscale (&gt;10^5)</td>
</tr>
</tbody>
</table>

Table 4.2 Preliminary study of the effect of lysozyme and heat on the bacteria.

The effect of temperature on the extraction was also investigated result in figure 4.8 shows that the temperature below 60°C shows little effect on the extraction of ATP from E.Coli. This efficiency increases after temperature reached above 60°C. The higher the temperature the greater efficiency of extraction. However the maximum temperature obtained from the water bath used was only 84°C. Greater efficiency could probably be obtained if heat above 84°C, by either using an oil bath or a heating block. For practical reasons, this work was carried out using the maximum water bath temperature unless where stated.

The strength of the lysozyme used has also an effect on the
Figure 4.9 The effect of increasing lysozyme concentration on the efficiency of the extraction.
Figure 4.10 The extraction of bacterial ATP using lysozyme.
extraction efficiency. Figure 4.9 show the linear relationship between lysozyme concentration and the production of light by the released ATP. The concentration of 0.8 grams/100ml was chosen for subsequent works.

Standard curve for the extraction of ATP from E.Coli by lysozyme is shown in figure 4.10.

A limit of detection of about $10^4$ cells/ml was obtain with a linearity from $10^4$ to $10^7$ cells/ml on a log-log plot.

During the course of this experiment the coil in the water bath was found to be clogged after a certain period of time, as the cell debris and protoplast produced accumulated in the tube. This phenomena can be taken as an indication of whether the lysis has taken place or not.

4.3.3 Extraction by Surfactants

Several surfactants were selected to represent each group i.e. anionic, cationic and non-ionic surfactants.

Table 4.3 showed the results obtained after 1 ml of 1% of each of the surfactants were added to 9 ml bacteria suspension in a sample vial. The mixture was incubated for about 10 minutes and then passed through the f.i.a. manifold and detected by firefly-luciferase reaction.
Detergents Bioluminescence Intensity (3 X 10^4)

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiling tris</td>
<td>243.30</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>3.83</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>5.33</td>
</tr>
<tr>
<td>Tween-80</td>
<td>3.67</td>
</tr>
<tr>
<td>Brij 35</td>
<td>6.17</td>
</tr>
<tr>
<td>DBSA</td>
<td>3.00</td>
</tr>
<tr>
<td>Mannitol</td>
<td>4.67</td>
</tr>
<tr>
<td>BDHA</td>
<td>11.39</td>
</tr>
<tr>
<td>Cetrimide</td>
<td>78.50</td>
</tr>
<tr>
<td>Cetyl pridinium bromide</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 4.3 The extraction of bacterial ATP by various surfactants.

Result in this table clearly shows that anionic (mannitol, DBSA) and non-ionic (Triton X-100, Tween 80, Brij 35) surfactants are not capable of extracting the bacterial ATP. With the exception of cetylpyridinium bromide, cationic surfactants are capable of extracting the ATP. The results also indicate the capability of cationic surfactant to attack the negatively charged peptide linkages in the bacterial cell walls giving to possible lysis of the bacteria to take place.
Although BDHA showed great extraction capability, it was found that it gave a slightly high blank reading and a very unstable baseline. This criteria could be due to phosphorescence of aqueous solution of this surfactant.

The extracting capability of cetrimide was further examined by adding 1% cetrimide solution to a serially diluted E.Coli suspension. The solution was incubated for about 10 minutes before the ATP determined.

The result shows that cetrimide is potentially a good extractant, while BDHA, though giving high reading found to give high blank reading as well reducing its advantage.

Triton X-100 has been previously used a extracting agent for somatic cells (Molin et al 1983), in this test it has not produce any extraction from the bacteria as predicted from an anionic surfactant.

Figure 4.11 shows the extraction of ATP from E.Coli by cetrimide.
Figure 4.11 The extraction of bacterial ATP using cetrimide.
Figure 4.12 F.i.a. manifold for cetrimide extraction

An experiment was designed to use cetrimide in a flow system by pumping through an extra channel in an f.i.a. manifold as shown in figure 4.12. Results in figure 4.11 show that in this case cetrimide failed to extract the ATP. The limit of detection was found to be lower by about 1 magnitude and also its sensitivity. This criteria could be due to high cetrimide:sample ratio in the manifold and also insufficient time for extraction to take place. Repeats of this experiment have also found to be unsuccessful in obtaining higher extraction efficiency.
4.3.4 Extraction By Nitric Acid

Results from this extraction is shown in table 4.4.

<table>
<thead>
<tr>
<th>Bacterial suspension</th>
<th>Unbuffered</th>
<th>Buffered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution no 1</td>
<td>3.5 X 10^4</td>
<td>201 X 10^4</td>
</tr>
<tr>
<td>Dilution no 2</td>
<td>--</td>
<td>37.5 X 10^4</td>
</tr>
</tbody>
</table>

Table 4.4 The preliminary study of nitric acid extraction.

The final pH of the unbuffered solution was 1.54 while the buffered solution was 7.17.

Results obtained show that there was no extraction observed in an unbuffered suspension, while in the pH adjusted suspension the extraction can be observed. This result clearly indicate that the need for pH adjustment for every extraction before bioluminescence reaction can be determined.

Figure 4.13 shows the curve obtained for the extraction of ATP from E.Coli carried out using nitric acid. Buffer with pH 8.49 was used to compensate for the lowering of pH by the acid.
Figure 4.12 The extraction of bacterial ATP using nitric acid.
4.3.5 Extraction by a Commercial Extractant (Picoex B)

Figure 4.14 shows the effect of the addition of the increasing amount of Picoex B to a 10ml bacterial suspension. Results obtained indicate that there was very little extraction occurred when small amount of this extractant was added and it increased as the amount added increased.

The amount of Picoex B was added was clearly inadequate for the extraction to take place as compared to the amount of bacterial suspension. A ratio of 2:100 dilution was used.

The curve for the extraction of the ATP by Picoex B is shown in figure 4.15 using non-f.i.a. measurement with a LKB luminometer. The f.i.a. determination showed no extraction when 200ul Picoex B was added to 10ml bacteria suspension.
Figure 4.14 The efficiency of ATP extraction using Picoex B.
Figure 4.15 The extraction of bacterial ATP using Picoex 3.
This investigation has shown several methods of extraction of bacterial ATP using flow injection analysis.

The use of ultrasound for the extraction of bacterial ATP has been described by Imshenetskii et al (1977). This method of extraction was easily incorporated into the f.i.a. system. It is also clean and no other material being introduced thus avoiding interference. However the need for a stopped-flow operation after certain period of time as required by the ultrasonic unit is a disadvantage especially if this method is used in a fully or semi-automated system. It was also discovered during this work that after certain period of time the temperature of the water where the extraction coil being immersed increased during the operation. This uneven and uncontrolled increase in the operating temperature is very undesirable as it could result in unpredictable extraction.

Despite these drawbacks the ultrasound has proved to be an instrument capable of extracting bacterial ATP. A more and efficient extraction was produced by a bigger ultrasonic generator i.e. cell disintegrator, while smaller ultrasonic generator i.e. ultrasonic bath unit has also proved to extract the ATP to a lesser extent. For an f.i.a. determination the ultrasound provide an excellent method as it introduces no fluid, hence no dilution involved in the
sample. Simple f.i.a. manifold can also be adapted as no additional channel involved.

Lysozyme extraction is another extraction method which can be easily adapted for an f.i.a. determination. Although the need for an extra channel in the f.i.a. manifold, it has an advantage as an enzyme its reaction is specific and not interfering with the ATP. And also the excess of this can easily be denatured in the heating process involved.

The use of surfactant as an extracting agent has not been very successful. Although cetrimide produces some degree of extraction, most other catalytic surfactants have not given any result. This could probably due to the slow action of these surfactant on the linkages between peptides chain in the cell wall. Furtherwork is suggested in this aspect to find any additional material to weaken the linkages before any involvement of these surfactant.

Although the use of acids as an extracting agent cannot be ruled out, work carried out has only been partly been successful. The low pH in the resulting solution is the main disadvantage of this method.

Picoex B, a commercial extracting agent has been successful in extracting the ATP. However, the limited amount of the substances available has limited its use in a continuous flow system such as f.i.a.. Probably a merging zone f.i.a. with two injection port would give a better result.
CHAPTER FIVE
MICROBIAL CONTAMINATION IN PHARMACEUTICAL PRODUCTS

5.1 Introduction

The determination of bacterial contamination in water and pharmaceutical products is of great importance to the pharmaceutical industry. Besides the need to adhere to regulations laid down by the authorities, the hazard to the health has also been of great concern. Several types of localised infections or septicaemia have been related to the presence of microorganisms in contaminated pharmaceutical products. *Pseudomonas aeruginosa* can cause severe eye infection if contaminated eye drops are used, while orally administered drugs if contaminated with *Salmonella* will cause stomach and bowel infections. Other than causing infection, bacterial contamination might also induce degradation of the products which would then reduce their effectiveness.

5.1.1 Sources of Contamination

The microbiological quality of pharmaceutical products is very much influenced by the environment in which they are processed and also by the raw materials used. Though some products are terminally sterilised in sealed containers, the debris and dead cells from the contaminated materials can still be toxic, especially if they are
derived from gram-negative bacteria.

Air usually contains spore forming bacteria (Baccilllus sp and Clostridium sp), non-spore forming bacteria (Staphyloccocus sp, Streptococcus Corynerbacter sp) and mould (Penicillium sp, Cladsporium sp, Aspergillus sp). These microorganisms are carried into the atmosphere by dust and droplets of moisture and are normally present in greater numbers in a dry compared to a damp atmosphere. During the process, these bacteria might be introduced into the products. Filtration is a method in which microbial contamination of the air is reduced in the process area using cellulose or glass wool filters. Chemical sprays and U.V are sometimes employed in room sterilization.

Water is another source of contamination in pharmaceutical products. Water is normally used for formulation in many products as well as for cooling and washing of raw materials. Though the water from the mains is normally free from contaminant such as E.Coli, other organism such as pseudomonas, flavobacter, chromabacter, bacillus subtilis, klebsiella aerogenes and enterobacter cloacae are sometimes present. Softened, deionised and distilled water might also contain microorganisms especially when faults occur in the equipment used to obtain this water. In some cases contamination occurs after these treatments.

Introduction of a contaminant through raw materials is a major problem. Products derived from animals such as
gelatine may be heavily contaminated with Salmonella or E.Coli. The British Pharmacopoeia(1980) requires such materials to be produced free from these organisms before they may be used in pharmaceutical preparations.

Synthetic raw materials normally contains less microbial contamination compared to natural products. Sterilisation procedures such as heat treatment, filtration, or irradiation are normally applied to raw materials before they are used in the pharmaceutical preparations.

The introduction of microbial contamination is not only confined to air, water and raw material, man is also a contributor to this contamination. Packaging and equipment have also been shown to contribute to microbial contamination in industrial processes.

5.1.2 Sampling

Drugs come in variety of dosage forms, normally they are in solution as syrups, tablets, suppositories or ointment form. In determining the contamination from different drugs product forms, a representative method of sampling has to be adopted. There are several sampling methods to suit different forms of drugs. Alwood (1973) has listed some of the methods for sampling and detection of the microbial contamination in different pharmaceutical products.
5.1.3 Regulations Concerning drugs contamination

Two types of tests are required by British Pharmacopoeia (1980) and U.S Pharmacopoeia (1980) to establish the presence of microorganisms in the pharmaceutical products.

i) Test of sterility.

ii) Test of microbial contamination.

Both tests require that no growth is observed in the plate count methods. For bacterial contamination, further tests are required to identify the organism present in the drug.
5.2 EXPERIMENTAL

5.2.1 Materials

ATP
ATP monitoring Reagent
Tris Buffer
Lysozyme
Cetrimide
Ephedrine hydrochloride
Nitrazepam
Phenacetin
Ethanol

5.2.2 General Procedures

ATP and ATP monitoring reagent were prepared as described in section 2.2.6.

Lysozyme and cetrimide were prepared as described in section 4.2.2.

Ephedrine hydrochloride, (0.1gm) was dissolved in distilled water (10ml) and 1 ml was then diluted to use.

Nitrazepam: - The drug was dissolved in ethanol (10ml). This solution was then diluted with water for use in the subsequent determination.
Phenacetin:— Phenacetin (1.0gm) was diluted in 99% ethanol (100ml). This solution was then diluted accordingly as required using distilled water.

Extraction using lysozyme:—The serially diluted bacterial suspension was prepared in the solution of the drugs prepared as described before. The extraction of bacterial ATP was then carried out using method and f.i.a. manifold as described in section 4.2.3.

Extraction using cetrimide:—1ml 1%cetrimide was added to 9ml solution of the drug with the bacteria, the mixture was then left for 15 minutes before the ATP detected using f.i.a. manifold described in section 4.2.3.

5.2.3 Methods

5.2.3.1 The Study of Bacterial Contamination in Ephedrine Hydrochloride.

The effect of ephedrine hydrochloride was studied by adding volumes of ephedrine (0.1%) to ATP solutions (9ml 10 M). The ATP was then determined using flow injection described in chapter 2.

In the study of bacterial contamination; to a serially
diluted E.Coli suspension(9ml), the ephedrine hydrochloride was added. The bacteria was then subjected to the extraction procedure using either lysozyme or cetrimide as described earlier.

5.2.3.2 The Study of Bacterial Contamination in Nitrazepam.

The effect of nitrazepam was studied by adding volumes of nitrazepam(0.1%) to ATP solutions (9ml 10^{-8}M). The ATP was then determined using flow injection described in chapter 2.

In the study of bacterial contamination; to a serially diluted E.Coli suspension(9ml), the nitrazepam was added. The bacteria was then subjected to the extraction procedure using either lysozyme or cetrimide as described earlier.

5.2.3.3 The Study of Bacterial Contamination in Phenacetin.

The effect of phenacetin was studied by adding volumes of phenacetin(0.1%) to an ATP solution (9ml 10^{-8}M). The ATP was then determined using flow injection described in chapter 2.

In the study of bacterial contamination; to a serially diluted E.Coli suspension(9ml), the phenacetin was added. The bacteria was then subjected to the extraction procedure using either lysozyme or cetrimide as described earlier.
5.3 RESULTS AND DISCUSSION

5.3.1 The investigation of microbial contamination in ephedrine hydrochloride

As reported by Jayaraman (1980) ephedrine hydrochloride has very little effect on ATP. Results in figure 5.1 show the effect of this drug on $1 \times 10^{-8}$ M ATP. The curve shows no effect of ephedrine on the ATP except for concentrations higher than 0.1% ephedrine. As expected, this behaviour could be due to high ionic strength of the solution which eventually deactivates the luciferase in its reaction with ATP.

Curves obtained when bacterial ATP was extracted from E.Coli in 0.01 and 0.001 % ephedrine hydrochloride solutions using cetrimide and lysozyme extraction methods are shown in figure 5.2 and 5.3 respectively.

Curves from these figures show a decrease in the amount of ATP detected in 0.1% as well as in 0.01% ephedrine solution for both methods of extraction.
Figure 5.1 The effect of ephedrine hydrochloride on ATP bioluminescence.
Figure 5.2 The effect of ephedrine on the ATP extraction by cetrimide
Figure 5.3 The effect of ephedrine on the ATP extraction by lysozyme
5.3.2 The investigation of microbial contamination in nitrazepam

Jayaraman (1980) also described the inhibition of 0.5 to 1% nitrazepam on the ATP bioluminescence. As the material was dissolved in methanol, the production of light by ATP might have been inhibited by the presence of methanol (see section 3.3.2).

This investigation also found no evidence of nitrazepam interference in the ATP bioluminescence at concentrations below 0.1% (see figure 5.4). A large reduction of the signal produced had been observed in concentrations greater than 0.1%. This inhibition could also be due to high ionic concentration which deactivates the enzyme luciferase by blocking its active sites. Complete inhibition was observed in a 1% nitrazepam solution.

The extraction of ATP by cetrimide was carried out in 0.01 and 0.001% nitrazepam solutions containing E.Coli is shown in figure 5.5, while extraction using lysozyme in similarly prepared nitrazepam solutions is shown in figure 5.6.

Results from both figures show no significant effect of nitrazepam on the extraction of ATP from E.Coli as compared to the extraction carried out in ephedrine hydrochloride solutions. Plate counts of the organisms in this drug solution has also not shown any significant reduction in
Figure 5.4 The effect of nitrazepam on ATP bioluminescence.
Figure 5.5 The effect of nitrazepam on extraction of ATP by cetrimide.
Figure 5.6 The effect of nitrazepam on extraction of ATP by lysozyme.
the number of bacterial counts in both water and nitrazepam.

Both lysozyme and cetrimide have not shown any blank reading in nitrazepam solution.

5.3.3 The investigation of microbial contamination in phenacetin

Phenacetin does not show any effect on the ATP bioluminescence at low concentration. However at higher concentration there is a tendency to inhibit the ATP reaction as shown in figure 5.7. It was also observed that the blank signal increased considerably at very high concentration i.e. 1% solution. The inhibition as shown by the plateau on the curve (see figure 5.7) occurs at a slightly lower concentration than 0.1% compared to nitrazepam. However the signal had not been completely reduced as experienced in 1% nitrazepam.

In the detection of bacterial ATP, figure 5.8 shows that the extraction of ATP from E.Coli by cetrimide (1%) can be carried out in low concentration of the drug. At higher concentration the inhibition can be slightly larger.

In the extraction using lysozyme, the same behaviour has been observed (figure 5.9). The extraction carried out in
0.01 and 0.001% phenacetin solutions also showed a slight reduction in the signal obtained, especially at 0.01% phenacetin.

Tests using agar plate count of the bacteria in water and in 0.01% phenacetin showed no reduction in the number of colonies formed.

No blank signal had been observed at low concentration, however, baseline signals and the blank reading were higher at high concentration (1%).
Figure 5.7 The effect of phenacetin on ATP bioluminescence.
Figure 5.8 The effect of phenacetin on extraction of ATP by cetrimide.
Figure 5.9 The effect of phenacetin on extraction of ATP by lysozyme.
5.4 CONCLUSION

This investigation has shown that the enumeration of bacteria (E.Coli) in pharmaceutical products can be carried out by ATP bioluminescence using flow injection analysis. All of the drugs investigated have not shown any effect on the release of the ATP and on pure ATP at low concentrations of the drugs. However at higher concentrations, some inhibition can be observed.

With the exception of ephedrine hydrochloride, these drugs have shown no interaction with the bacteria. In the case of ephedrine hydrochloride the number of colonies formed in an agar plate count was reduced by nearly 50%. Considering the effect of ephedrine on pure ATP, this criteria suggested that the reduction was mainly due to the interaction between drug and the bacteria rather than the inhibition of the ATP reaction by the drug itself. Due to this behaviour, it is suggested that any determination of bacterial contaminations using these procedures be carried out as soon as possible and the readings should also be compensated for the loss of the bacteria during storage and analysis.

Due to the high detection limits of these methods, the use of these procedures to determine the sterility and the microbial contamination of the drugs is not possible. This
detection limit can only be achieved if a better extraction is used and a more sensitive detection instrument is available. For detection as little as 1 organism/ml is required by these tests, a detection limit of pure ATP of up to 1 \times 10^{-13} M is required (taking 1 \times 10^{-10} M ATP per cell). In this work attempts to achieve these detection limit have not been successful.
CHAPTER SIX
6.1 GENERAL CONCLUSION

This investigation shows that ATP can be easily determined using flow injection analysis. In a typical bioluminescence reaction, a flash is normally produced which then declines slowly. F.i.a. provides an excellent tool for measuring this transient light reproducibly. The need for a critical mixing and timing can also be eliminated in an f.i.a. system.

The other advantage is that the inclusion of air bubbles have not affected any signal as experienced with other methods of detection such as colorimetry and fluorimetry.

The detection limit has also been found to be comparable to the non-f.i.a. method as described by the manufacturer. This detection limit could well have been improved if the detector i.e the liquid scintillation detector, used in this work was in perfect working condition.

Bacterial enumeration using ATP bioluminescence have been carried out by several workers (Chappelle and Levin 1963; Thore et.al 1975) using different methods for the extraction of the ATP such as boiling tris, PCA and TCA. In this work, several methods have been investigated for possible use in a flow system.
Ultrasonic extraction can be seen as a good proposition for used in a flow injection system. This method can be easily adapted by passing the sample through a coil immersed in the ultrasonic bath. However, a small ultrasonic bath has been found to be inadequate in releasing the ATP from the cells. The need for stoppages and the increase in the operating temperature have made this method unsuitable for used in a fully automated system. Headache has also been experience cause by the sound generated by these units.

Lysozyme and cetrimide were found to be the simplest and the most pleasant extraction method to be adapted for an f.i.a. determination. Lysozyme can be easily adapted for f.i.a. analysis and available in abundance and harmless. The solution has also not shown any effect on pure ATP and neither does it give any blank reading. While in the case of cetrimide, it can be used in a simple form as a solution of 1% strength added to the solution containing the bacteria. The solution has also not given any blank or affecting the ATP. The only drawback is that it did not work when used in a flow system, probably due to slowness of its reaction on the cell walls. However this method has proved to be capable of extracting the microbial ATP and adaptable to an f.i.a. system.

Other methods investigated were found to unsuitable for use in an f.i.a. system. It either needed a complicated manifold or sometimes extraction was inefficient. The limited amount of the extractant available as in the case
of the commercial enzyme has also had to be taken into account.

Several other methods reported by several workers using organic solvents have not been investigated as our investigation shows that these solvents inhibit the ATP bioluminescence even in small amount. The need for separation between organic and the aqueous phase after the extraction has also made these methods unattractive for use in an f.i.a. determination.

In the determination of microbial contamination in pharmaceutical products, ATP bioluminescence and f.i.a. offer a quick, simple and easily automated system. The level of detection achieved in this work indicates that the test for drug sterility is not possible. Sterility can be defined a total absence of the microorganism, in this method, the organism can only be quantified up to around $10^4$ cells/ml depending on the extraction method used. However, with this limit of detection there are other possible uses for this methods such as the test for the interaction of the drug and microorganism and also test for efficiency of anti-microbial drugs.

The other problem regarding the determination of microbial contamination in drugs, is the effect of the drug on the activity of the microorganisms as shown by the ephedrine hydrochloride.
In conclusion this investigation has shown that bacterial
enumeration can be carried out using ATP bioluminescence
with flow injection analysis. The detection limit for the
numbers of detectable bacteria can probably be improved if
a more efficient extractant could be found and more
sensitive detector used. Its convenience, quick and
simplicity have made this work attractive.
REFERENCES


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APPENDIX I

ACRONYMS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>E</td>
<td>enzyme (luciferase)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>FMNH₂</td>
<td>reduced form of FMN</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>LH₂</td>
<td>luciferin in reduced form</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced form of NAD</td>
</tr>
<tr>
<td>PCA</td>
<td>perchloric acid</td>
</tr>
<tr>
<td>PPI</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
</tbody>
</table>
APPENDIX II

ATP: Sigma (London) Chemical Co. Ltd., Poole, Dorset.


ATPase: Sigma (London) Chemical Co. Ltd., Poole, Dorset.


Cetrimide: BDH Chemicals Ltd., Atherstone, Worcs.

Cetylpyridinium chloride: Sigma (London) Chemical Co. Ltd., Poole, Dorset.

Ephedrine hydrochloride: BDH Chemicals Ltd., Atherstone, Worcs.

Lysozyme: Sigma (London) Chemical Co. Ltd., Poole, Dorset.

Nitrazepam: Roche Products Ltd., Welwyn Garden City, Herts.

Nitric acid: Fisons Scientific Apparatus, Loughborough, Leics.

Phenacetin: BDH Chemicals Ltd., Atherstone, Worcs.
Pyrophosphatase: Sigma (London) Chemical Co. Ltd., Poole, Dorset.

Sodium pyrophosphate: BDH Chemicals Ltd., Atherstone, Worcs.

Tris: Fisons Scientific Apparatus, Loughborough, Leics.

Triton X-100. BDH Chemicals Ltd., Atherstone, Worcs.
APPENDIX III

MICROBIAL PREPARATIONS

i) Nutrient Broth

Beef extract 3g
Peptone 5g

Method: Dissolve the ingredients in 1 liter of distilled water. Autoclave at 121 C for 15 minutes Final pH 6.8.

ii) Nutrient Broth No. 2.

formula:
beef extract 10g
peptone 10g
sodium chloride 5g

Method: Dissolve the ingredient in 1 liter water, adjust reaction so that pH after autoclaving will be around 7.5, dispensed as required and autoclave at 121 C for 15 minutes.

iii) Nutrient Agar

formula:
beef extract 3g
peptone 5g
agar 15g
Method: Add ingredient to 1 liter of distilled water, heat to boiling until solution is complete, cool to 50-60 C and adjust reaction so that pH after sterilisation will be around 6.8 to 7.0 and dispensed as required.

iv) Ringer solution.

formula:

sodium chloride 9g
potassium chloride 0.42g
calcium chloride 0.48g
sodium bicarbonate 0.20g

Method: Dissolved ingredients in 1 liter distilled water and sterilize at 121 C for 10 minutes.